University of Alberta

Aging Increases Cytochrome P450 4A Modulation of α_1-Adrenergic Vasoconstriction

by

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Abstract

Dysfunction of the peripheral vasculature may underlie the aging-related increase in the risk of developing cardiovascular disease. In vascular smooth muscle cytochrome P450 4A (CYP4A) enzymes ω-hydroxylize arachidonic acid to form the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE). CYP4A-dependent production of 20-HETE contributes to increased vasoconstriction noted in spontaneously hypertensive rats; however, the role of vascular CYP4A/20-HETE in aging is unknown. *We hypothesized that aging increases CYP4A modulation of vasoconstriction in resistance arteries from male and female rats.* In agreement with our hypothesis, CYP4A modulation of α_1-adrenergic vasoconstriction was increased in aged (relative to young) male rats, and aged ovarian hormone-deficient (relative to aged intact and estrogen-replaced) female rats. Interestingly, CYP4A inhibition did not affect endothelin-1- or pressure-induced constriction. These data suggest that aging specifically increases α_1-adrenergic-dependent activation of CYP4A/20-HETE. Further study may allow for the development of novel therapeutic interventions for the prevention of cardiovascular disease in aging men and women.

Dedication

This Thesis Is Dedicated To My Friend & Mentor Dr. Sandra Davidge

Dear Sandy,

Thank you for your caring support and encouragement over the last four years. You were my main source of motivation for every step of this process including daily experiments and the composition of this thesis. I have benefited greatly from your knowledge, insight and enthusiasm. I am also very grateful for all of the compassion you have shown toward me and my family. Your understanding has eased the weight of the last few years dramatically. Above all though, thank you for your friendship. It has made working with you truly exceptional.

Sincerely,

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List of Abbreviations

19-HETE	19-hydroxyeiocsatetraenoic acid
20-HETE	20-hydroxyeicosatetraenoic acid
ACh	acetylcholine
AA	arachidonic acid
ANOVA	analysis of variance
BK_{Ca}	large conductance calcium-activated potassium channel
cDNA	complementary deoxyribonucleic acid
COX	cyclooxygenase
CVD	cardiovascular disease
CYP	cytochrome P450 enzyme
DDMS	N-methylsulfonyl-12,12-dibromododec-11-enamide
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
EDHF	endothelium-derived hyperpolarizing factor
EET	epoxyeicosatrienoic acid
ET-1	endothelin-1
ET_A	endothelin A receptor
ET_B	endothelin B receptor
HEPES-PSS	HEPES-buffered physiological saline solution
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate

mRNA	messenger ribonucleic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2	nitrogen dioxide
NOS	nitric oxide synthase
OVX	ovariectomized
OVX-E	ovariectomized and estrogen-replaced
PLA_2/C/D	phospholipase A_2/C/D
PGI_2	prostacyclin
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
TEMED	N,N,N',N'-tetramethylethylenediamine
WKY	Wistar-Kyoto

CHAPTER I – INTRODUCTION

1. Overview

Cardiovascular disease is a leading cause of death among Canadians.[1] Moreover, it accounts for the majority of deaths world-wide in both developed and developing nations.[2,3] Several of the risk factors for developing cardiovascular disease are modifiable including obesity, physical inactivity, and smoking.[4] Aging, however, is the major unremitting source of risk for the development of cardiovascular disease. For both men and women, advancing age is accompanied by increased rates of acute myocardial infarction,[5] atherosclerosis,[6] congestive heart failure,[7,8] and systolic hypertension.[9,10] Interestingly, aging-related dysfunction of resistance arteries underlies the development of these conditions. This introduction will outline aging-related functional changes in the peripheral resistance vasculature with the major focus being on vascular cytochrome P450 4A (CYP4A) enzymes that catalyze the formation of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE).

2. The Resistance Vasculature

Resistance arteries are, by definition, arteries whose diameters collectively determine peripheral resistance, and thus, contribute substantially to blood pressure.[11] According to Poiseuille's law, the resistance through a tube is inversely proportional to the radius of the tube to the fourth power.[11] Thus, a small reduction in arterial diameter can cause a marked increase in total peripheral resistance. Therefore, the diameters of resistance arteries are tightly regulated by neural, endocrine, and local stimuli that can act on both the endothelial cells lining the lumen of the arteries as well as the underlying smooth muscle cells.[11] The following section will briefly outline the major mechanisms that play a role in the regulation of vascular function with a focus on the specific pathways relevant to this thesis.

Vascular endothelial cells generate a variety of vasoconstrictors and vasodilators that act collectively to affect vascular smooth muscle tone (Figure 1.1). There are three major vasodilators that originate in the endothelium: nitric oxide (NO), prostacyclin (PGI_2), and a group of molecules referred to as the endothelium-derived hyperpolarizing factor (EDHF).[12] NO is an extremely potent vasodilator formed by

nitric oxide synthases (NOSs).[13] NOS enzymes are heme-containing proteins that catalyze the formation of NO through the conversion of L-arginine to L-citrulline.[14,15] Three isoforms of NOS have been identified: NOS-1 (neuronal NOS; nNOS), NOS-2 (inducible NOS; iNOS) and NOS-3 (endothelial NOS; eNOS).[13] Under normal physiological conditions, eNOS is the endothelial source of NO.[13] Subsequent to its formation, NO causes relaxation of vascular smooth muscle cells by activating soluble guanylate cyclase and thus increasing intracellular cyclic guanosine monophosphate levels.[16] This molecule then acts to cause vasodilation through a wide variety of mechanisms that combine to reduce intracellular calcium concentrations. reviewed in [16] The second major vasodilator, PGI_2, is synthesized by cyclooxygenase (COX) enzymes from the important lipid precursor arachidonic acid (AA).[12] AA, a 20 carbon ω-6 fatty acid, also serves as a substrate for the production of many other vasoactive molecules (see below). The final major mechanism for endothelium-dependent vasodilation is the EDHF pathway. EDHF refers to all substances that result in the hyperpolarization of vascular smooth muscle (with the exception of NO and PGI_2). EDHFs have been reported to consist of several substances including potassium ions, hydrogen peroxide, and AA metabolites produced by cytochrome P450 (CYP) enzymes called epoxyeicosatrienoic acids (EETs).[12] However, the precise nature of the EDHF varies by vascular bed and species.[12]

Endothelium-derived vasoconstrictors are also important in the regulation vascular smooth muscle tone. Indeed, endothelial cell production of thromboxane A_2, a vasoconstrictor AA metabolite formed by COX enzymes, and endothein-1 (ET-1), a potent peptidergic vasoconstrictor, elicit powerful contraction of vascular smooth muscle cells.[17,18] ET-1 induces constriction by binding to its receptors (ET_A and ET_B receptors) on vascular smooth muscle cells.[17] ET_A receptors, however, are believed to mediate the majority of the vasoconstrictor effects of ET-1.[19-21] It is interesting to note that ET-1 binding of ET_B receptors on the endothelium results in vasodilation through activation of eNOS;[17] however, this thesis will focus on the vasoconstrictor actions of ET-1 as they relate to aging and the CYP4A/20-HETE pathway (see below).

Vasoactive molecules are also produced by the nervous and endocrine systems. Specifically, parasympathetic stimulation of the peripheral arteries results in vasodilation due to the release of acetylcholine (ACh) while the sympathetic system largely results in vasoconstriction due to the release of norepinephrine.[22] In addition, hormonal release of epinephrine and angiotensin II can also cause vasoconstriction of the peripheral arteries.[11]

Finally, substances produced within the vascular smooth muscle can act intracellularly to influence vascular tone. Of specific interest to this thesis is that agonist-induced activation of vascular smooth muscle results in the liberation of AA from the plasma membrane. Free AA is then readily metabolized by three classes of enzymes including the COX and CYP enzymes discussed above.[12] AA is also metabolized by lipoxygenase enzymes to form hydroperoxyeicosatetraenoic acids (HPETEs) and leukotrienes.[12] Although, COX and lipoxygenase products are of significant importance in the regulation of vascular function, this thesis will focus on AA metabolism by CYP enzymes. As mentioned previously, endothelial CYPs largely produce vasodilatory EETs that may act as EDHFs. However, the CYP enzymes expressed in vascular smooth muscle generate 19- and 20-HETE in addition to EETs.[23] While EETs and 19-HETE have vasodilatory actions, 20-HETE is a potent vasoconstrictor.[23,24]

Activation of vascular smooth muscle by vasoconstrictors including ET-1 and adrenergic catecholamines results in vasoconstriction through a relatively well-defined pathway.[25] Binding of these compounds to their respective G-protein coupled receptors results in the activation of phospholipase C (PLC). PLC then cleaves phosphatidylinositol bisphosphate to form diacylglycerol and inositol triphosphate (IP_3). Subsequently, IP_3 binds to its receptor on the sarcoplasmic reticulum to cause calcium release through both IP_3 and ryanodine receptor channels. Increased intracellular calcium allows for myosin light chain phosphorylation by myosin light chain kinase and subsequent vasoconstriction due to actin-myosin crossbridging.

Importantly, PLC, as well as other phospholipases, can result in the liberation of AA from the plasma membrane and the subsequent metabolism of this lipid. In this way, agonist-induced vasoconstriction can activate CYP4A production of 20-HETE. The relative importance of these pathways in aging is a primary focus of this thesis.

3. Aging & the Resistance Vasculature

Aging profoundly alters both endothelial and smooth muscle cell function. Generally, aging results in a reduction in endothelium-dependent vasodilation[26] and, accordingly, increased vasoconstrictive influences in the vascular environment. In addition to changes in vascular reactivity, vascular remodeling occurs with aging. This section will summarize vascular changes in aging.

In large conductance arteries, it is well known that aging is associated with significant vascular remodeling and reduced arterial compliance;[27] however, less information is available regarding the possible aging-induced structural changes in resistance arteries. In humans, the effects of aging on arteries are vascular bed-dependent. Indeed, carotid arteries display an age-related increase in stiffness while femoral arteries do not.[28] In mesenteric resistance arteries from rats, aging results in arterial hypertrophy, but does not affect arterial compliance.[29,30] These data highlight the need for future studies to further elucidate the effects of aging on the structural properties of the peripheral vasculature. It is known, however, that aging affects both vasodilatory and vasoconstrictor properties of the vasculature.

3.1. Vasodilation & Aging

Aging is associated with reduced NO-dependent vasodilation in peripheral arteries from animals and humans.[26,31,32] This may result from an aging-related increase in oxidative stress within the vasculature that acts to reduce the availability of NO.[15] Oxidative stress is defined as an imbalance between pro- and anti-oxidant factors favoring a shift toward a pro-oxidative state.[33] Indeed, in the presence of the potent pro-oxidant molecule superoxide, NO is scavenged to form the powerful oxidant, peroxynitrite.[34] Importantly, this

reaction reduces the amount of NO available to cause vasodilation. Basal superoxide formation in mesenteric arteries from aged rats is increased relative to young rats.[35] Furthermore, aging reduces the expression of superoxide dismutase (SOD). SOD breaks down superoxide to form hydrogen peroxide. In humans, SOD content in the abdominal aorta as well as the middle cerebral, basilar and common carotid arteries was negatively correlated to advancing age.[36] Furthermore, treatment of aged rats with the SOD mimetic tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), normalized aging-induced changes in vasoconstriction and NO-dependent vasodilation.[37] These data therefore suggest that increased superoxide formation in aging may reduce the bioavailability of NO thus decreasing its capacity to induce vasodilation.

Both NO and peroxynitrite can oxidize intracellular proteins. NO selectively modifies cysteine residues to form S-nitrosocysteine.[38] These nitrosylation (addition of an NO group) reactions may account for the soluble guanylate cyclase-independent actions of NO in vascular smooth muscle.[38] Peroxynitrite, however, can result in the direct nitration (addition of an NO_2 group) of amino acids (e.g. cysteine, methionine and tryptophan), and of transition metal centres (containing iron, copper and manganese ions).[39] Furthermore, degradation products of peroxynitrite can have similar actions.[39] For example, reaction of peroxynitrite degradation products such as the hydroxyl or NO_2 radicals with tyrosine residues results in nitration of these residues and the formation of nitrotyrosine.[39] Nitrotyrosine immunostaining is frequently used as a marker of oxidative stress in vascular tissues. Indeed, nitrotyrosine staining is increased in aortae from aged rats.[40] In addition, nitrotyrosine levels in endothelial cells from brachial arteries of older men (63 $\pm$ 1 years) are elevated relative to those from younger men (23 $\pm$ 1 years).[41] This increase was accompanied by reduced endothelium-dependent vasodilation.[41] Overall, aging is associated with increased production of oxidants that can act to damage cellular proteins and disrupt vascular function.

Aging can also affect COX- and EDHF-dependent vasodilation. As mentioned above, ACh is an important vasodilatory neurotransmitter. It causes vasodilation by binding to its receptors on endothelial cells and activation of the NO, PGI_2 and EDHF pathways.[12] Sensitivity to ACh or its mimetic (methacholine) is reduced in aortae[42] and resistance arteries of aging rats[42,43] as well as coronary[44] and brachial arteries in older humans.[45] COX-dependent production of PGI_2 has been reported to be unaffected by aging in skeletal muscle arterioles from rats; although aging did reduce the expression of messenger ribonucleic acid (mRNA) for the COX-1 isoform in these animals.[46] Our laboratory has demonstrated that inhibition of the COX-2 isoform reduces vasoconstriction in isolated mesenteric arteries from aged rats indicating that aging results in COX-dependent formation of a vasoconstrictor.[47] Accordingly, aging may reduce COX-dependent vasodilation. Various studies have reported reduced[48,49] or normal[26] contributions of EDHF to ACh-induced vasodilation in aging. Likely, the effects of aging on EDHF-dependent vasodilation depend on the identities of the specific EDHF in the vascular bed in question. Overall, aging is associated with impaired endothelium-dependent vasodilation that may result from alterations in the synthesis of NO, PGI_2, and EDHF.

3.2. Vasoconstriction & Aging

Aging also has profound effects on vasoconstrictor pathways. Indeed, aging is associated with increased plasma ET-1 levels in men and women.[50,51] Vascular ET-1 expression has been reported to be elevated[52] or reduced [53] in aging rats depending on vascular bed. The effects of aging on ET_A gene expression have not been determined in all vascular beds; however, ET_A expression has been shown not to be affected by age in skeletal muscle arterioles from rats.[54] Thus, the role of the ET-1/ET_A pathway in aging remains to be fully elucidated.

Several studies have examined the effects of aging on adrenergic vasoconstriction; however they have not yielded consistent results. Systemic treatment of aged monkeys with norepinephrine, or the α_1-adrenergic agonist phenylephrine, reduced total peripheral resistance to a lesser degree than in younger animals.[55] Furthermore, aging is associated with reduced adrenergic responses as measured by subcutaneous arterial constriction[56] and reductions in forearm blood flow[57] in humans. By contrast, aging has also been reported to *increase* α-adrenergic vasoconstriction in men.[58] Similarly, in rats, aging decreases adrenergic sensitivity in tail arteries[59] while it increases norepinephrine-dependent vasoconstriction in skeletal arterioles[60] and phenylephrine-dependent vasoconstriction in mesenteric arteries.[61] The data regarding α_1-adrenergic receptor expression in aging are similarly complex. In rats, the expression of α_1-receptor subtypes α_{1A}, α_{1B}, and α_{1D} is reported to be unaffected by aging in aortae[62] and mesenteric arteries.[63] However, another study reported that aortic α_{1B}-receptor expression was reduced in aged rats.[64] In humans, aging results in increased α_{1A} and α_{1B} receptor expression in mammary arteries.[65] Accordingly, further study is necessary to determine the overall role of α-adrenergic signalling in aging.

Although aging may have profound effects on other vasoactive pathways, the CYP4A/20-HETE pathway has been reported to modulate vasoconstriction induced by phenylephrine[66] and ET-1[67] and, as described above, both of these pathways are altered in aging. Therefore, *this thesis will focus on CYP4A modulation of α_1-adrenergic- and ET-1-dependent vasoconstriction in aging.*

4. Cytochrome P450 (CYP) Enzymes

CYP enzymes are heme-containing monooxygenases that were initially found to facilitate the biotransformation of exogenous chemicals in the liver.[68] In these reactions, nicotinamide adenine dinucleotide phosphate (NADPH) acts as a reducing agent to allow CYP enzymes to oxidize the foreign chemical resulting in more polar

end products that can be filtered through the glomerulus and excreted in the urine.[68] Subsequent investigations have identified over 500 genes that code for CYP enzymes in prokaryotic and eukaryotic species including humans.[69] Furthermore, CYP enzymes have now been shown to oxidize numerous endogenous lipophilic substances such as fatty acids, steroids and vitamins in extrahepatic tissuses.[23] In the vasculature, it has been well-characterized that CYP enzymes metabolize AA to form a variety of vasoactive molecules. Specific to the interests of this thesis, ω-hydroxylation of AA by CYP4A results primarily in the generation of the potent vasoconstrictor 20-HETE.[23] Moreover, the CYP4A/20-HETE pathway has been shown to be important in the modulation of vasoconstriction in resistance arteries from a variety of vascular beds in both normal and pathological states.[23] The history of the characterization of vascular CYP4A production of 20-HETE is detailed in section 4.3. We will begin, however, by discussing the nomenclature of the CYP enzymes and their mechanisms of action.

4.1. Nomenclature for CYP Enzymes

The nomenclature for CYP enzymes is complex. CYP enzymes are categorized according to family (sharing at least 40% amino acid homology) denoted by an Arabic numeral, subfamily (sharing at least 55% homology) denoted by a letter, and specific isoform denoted by a second Arabic numeral.[69] Names of CYP protein, mRNA and complimentary DNA (cDNA) are written in capitals.[69] Names of CYP genes are capitalized and italicized with the exception of mouse *Cyp* genes that are written in small letters.[69] Thus, all enzymes in the CYP4 family are fatty acid hydroxylases while enzymes from the CYP4A subfamily primarily hydroxylize fatty acids at the final (ω) or ω-1 carbon atoms.[23] The letters denoting CYP4A isoforms are species-specific with CYP4A1, CYP4A2, CYP4A3 and CYP4A8 expressed in rats, CYP4A4, CYP4A5, CYP4A6 and CYP4A7 expressed in rabbits, CYP4A10, CYP4A12, CYP4A14 expressed in mice, and CYP4A9 and CYP4A10 expressed in humans.

4.2. CYP4A-Mediated Reactions

CYP enzymes act in conjunction with the flavoprotein NADPH-cytochrome P450 reductase. Specifically, protons are transferred from NADPH to this enzyme allowing the subsequent donation of electrons to the CYP reaction (Figure 1.2). In this way, NADPH serves as a crucial electron donor to CYP-dependent metabolism. As depicted in Figure 1.2, the ferric form of the CYP enzyme (containing Fe^{3+}) binds to the substrate. The complex is then reduced to the ferrous form (containing Fe^{2+}) by an electron from the NADPH-cytochrome P450 reductase reaction. Subsequent reaction with molecular oxygen and addition of another electron results in a ferrous CYP-substrate complex bound to activated oxygen that then dissociates to form water and the oxidized substrate as well as re-generation of the ferric enzyme.[68]

The origin of the second electron is not entirely known. Cytochrome b_5 is a ubiquitously expressed heme-containing protein that acts to transfer electrons.[70] AA metabolism by CYP enzymes from the 4A family has been shown to be dependent on the presence of cytochrome b_5.[71] Furthermore, cytochrome b_5 increases the rate of AA metabolism in liver microsomes from pigs.[72] These data suggest that cytochrome b_5 may provide electrons to the CYP reaction; however, the mechanisms through which this may occur are unclear. Indeed, cytochrome b_5 may act as a direct electron donor, it may form a complex with the CYP-substrate entity, or it may act to reduce CYP uncoupling.[70] NADPH oxidation and substrate oxidation are not completely coupled in CYP-dependent reactions depending on the substrate and the CYP isoform present.[70] Thus, CYP-dependent reactions can produce superoxide in addition to oxidized substrate.[73] Addition of cytochrome b_5 to liver microsomes reduces hydrogen peroxide formation (the product of superoxide dismutation) without increasing NADPH consumption.[74] Accordingly, although cytochrome b_5 is essential to NADPH cytochrome P450 reductase

and CYP-dependent metabolism, its mechanisms of action remain to be determined.

4.3. Initial Investigations into the Roles of CYP4A Enzymes

In the early 1980s it was reported that enzymes within liver microsomes from rats were capable of NADPH-dependent oxidation of AA.[75] Both ω and ω-1 hydroxylation products were detected. CYP enzymes were hypothesized to catalyze these reactions.[75] In 1981, Morrrison and Pascoe reported that kidney cortical microsome monooxygenenases produced 19- and 20-HETE when incubated with AA.[76] This was the first report of renal production of 20-HETE. Furthermore, in these preparations, 19- and 20-HETE were the primary products of AA (relative to those produced by COX and lipoxygenase).[76] A schematic outline of CYP4A-dependent 20-HETE formation in depicted in Figure 1.3

The interest in renal CYP enzymes was rekindled almost 10 years later with several reports of increased CYP activity and expression in the kidneys of SHR animals relative to normotensive controls. Firstly, when incubated with radiolabled [^{14}C]-AA, cortical microsomes from SHR exhibited more AA metabolite formation during the development of hypertension relative to their age-matched, normotensive Wistar-Kyoto (WKY) counterparts.[77] Production of these metabolites was susceptible to the non-specific CYP inhibitor β-diethyl-aminoethyldiphenylpropylacetate (SKF-525A) but not the COX inhibitor indomethacin.[77] Furthermore, treatment with heme arginate reduced blood pressure and renal CYP activity in SHR but not WKY animals.[78] Heme arginate treatment in these animals induced heme oxygenase activity, and thus, reduced CYP activity through degradation of heme.[78] Similarly, stimulation of heme oxygenase with stannous chloride ($SnCl_2$) also prevented the development of hypertension in young SHR.[79,80] Treatment of adult SHR animals, however, did not reduce CYP metabolism and thus did not affect blood pressure.[23, 80] In 1991 it was demonstrated that renal expression of

mRNA for CYP4A2 was four times higher in SHR than WKY animals at 28 weeks of age.[81] However, the levels were similar at 16 weeks of age.[81] Collectively, these data suggested that renal CYP-dependent metabolism of AA was responsible for the generation of hypertension during development in the SHR.

Later, it was shown that 20-HETE is the primary product of CYP activity in the kidney.[82] In addition, although production of 20-HETE was increased in SHR preparations, its synthesis was also detected in kidneys from normotensive WKY animals.[82] Similarly, CYP4A mRNA and protein was detected in liver and kidneys of normotensive rats.[83] Again, ω-hydroxylation was reported to be the primary reaction catalyzed by these enzymes.[83] These data indicated that CYP4A-dependent production of 20-HETE may also be important in normotensive animals.

The involvement of CYP enzymes in vascular function was first explored in renal arcuate arteries from dogs.[84] Myogenic constriction normally develops in these arteries as the intraluminal pressure is increased from 80 to 160 mmHg. Generation of this tone was partially inhibited by the CYP inhibitors SKF 525A and ketoconazole but not by indomethacin. Furthermore, microsomes from these arteries produced 20-HETE and treatment with SKF 525A or ketoconazole inhibited its synthesis. This important study suggested that CYP production of 20-HETE acts to support myogenic tone in renal arteries from normotensive animals. Subsequently, it was shown that ketoconazole prevented myogenic tone development in interlobular arteries as well as proximal and distal afferent arterioles from SHR, WKY, and the inbred control strain Wistar-Lewis.[85] In addition, this study demonstrated that preglomerular arteries from SHR animals produced more 20-HETE than arteries from normotensive WKY and Wistar-Lewis animals.[85] Combined, these data suggest that CYP activity contributes to pressure-dependent vasoconstriction in renal arterioles from normotensive animals and that

increased ω-hydroxylase activity in the SHR contributes to hypertension in these animals.

A study conducted by Harder *et al.* in 1994 specifically identified CYP4A protein in the cerebral microvasculature of adult cats.[86] Furthermore, addition of exogenous 20-HETE resulted in vasoconstriction of pial arteries.[86] This study also suggested that inhibition of endogenous CYP-dependent 20-HETE formation increases the open probability of potassium channels. This indicated that CYP enzymes in these arteries may produce 20-HETE which then contributes to basal vascular tone by inhibiting potassium channels and thus vascular hyperpolarization. Similarly, other studies have reported that inhibition of CYP-dependent ω-hydroxylation reduces pressure-induced vasoconstriction in the renal arterioles of the rat.[87,88] These studies indicated that CYP4A-dependent production of 20-HETE is an important endogenously active vasoconstrictor pathway in both renal and cerebral arteries. The specific mechanisms of action of CYP4A and 20-HETE will be discussed in the following section.

5. CYP4A and 20-HETE in Vascular Function

In recent years, it has been well characterized that endogenous CYP4A-dependent production of 20-HETE contributes to vasoconstriction induced by increased intraluminal pressure and by various vasoconstrictor agonists in renal, cerebral, coronary and systemic resistance arteries.[23] Moreover, endogenous 20-HETE acts as an intracellular messenger to cause vasoconstriction through the inhibition of several cation channels.[86,89,90] Interestingly, exogenous 20-HETE has been reported to cause both vasodilation[91] and vasoconstriction.[86] This section will detail the findings regarding the involvement of CYP4A and 20-HETE in vasoreactivity to date.

5.1. Pressure-Induced Constriction

As mentioned above, inhibition of CYP enzymes reduces myogenic tone development in renal arterioles from dogs[84] and Sprague-Dawley rats[87] (see

section 4.3). In the latter study, afferent arterioles from rats developed an 8 ± 1 % reduction in arterial diameter as the intraluminal pressure was increased from 80 to 160 mmHg. Treatment with the CYP inhibitor 17-octadecynoic acid (17-ODYA) prevented this constriction and resulted in a 7 ± 3 % increase in diameter over this pressure range.[87] Thus it would seem that CYP products are entirely responsible for the generation of myogenic tone in these arteries. The authors tested the specificity of 17-ODYA and found that it inhibited CYP-dependent production of 20-HETE as well as the production of EETs by other CYP enzymes. Using a more specific inhibitor N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) it was later confirmed that myogenic tone development in renal arterioles is dependent upon on CYP-dependent ω-hydroxylation.[92]

In rat cerebral arteries, myogenic tone development was reduced, but not completely abolished, by treatment with DDMS or either of the physiological antagonists of 20-HETE, 20-HEDE (20-hydroxyeicosa-6(Z),15(Z)-dienoic acid) or 15-HETE (15-hydroxyeicosatetraenoic acid).[93] Interestingly, this study also found that arterial 20-HETE production was increased by elevated intraluminal pressure. These data therefore suggest that 20-HETE contributes to myogenic constriction in the cerebral vasculature as well. In contrast, pressure-induced vasoconstriction is not affected by DDMS treatment in coronary arteries from wildtype mice. However, in eNOS knockout mice, DDMS treatment did reduce myogenic constriction in these arteries suggesting that, in the absence of eNOS-dependent production of NO, ω-hydroxylase activity, and thus 20-HETE production, are up-regulated and contribute to vasoconstriction.[94] CYP4A has also been shown to modulate myogenic constriction in the systemic resistance vasculature. In hypertensive, but not normotensive Dahl Salt-Sensitive rats, inhibition of CYP4A abolished pressure-induced constriction in gracilis muscle arterioles.[95] Furthermore, treatment with DDMS or 20-HEDE abolished myogenic tone development in mesenteric arteries from normotensive male Sprague-Dawley rats.[96] It would

seem then, that the CYP4A/20-HETE pathway is important in myogenic tone development in a variety of vascular beds.

5.2. Agonist-Induced Constriction

CYP4A inhibition with DDMS reduces arteriolar sensitivity to several vasoconstrictors including Ang II,[97] the thromboxane A_2 mimetic U46619,[98] and ET-1 in the renal vasculatue[99] as well as Ang II[100] and phenylephrine sensitivity in mesenteric arteries.[66] The majority of the data, however, are regarding ET-1-and phenylephrine-dependent vasoconstriction. Furthermore, these two constrictor pathways are of specific interest in this thesis.

ET-1 is a powerful inducer of 20-HETE (but not 19-HETE) formation in renal microsome preparations from rats.[99] Moreover, inhibition of ω-hydroxylation reduces ET-1 sensitivity in preglomerular arterioles.[67,101] In addition, DDMS significantly reduces the ET-1-dependent increase in intracellular calcium concentrations.[101] These data suggest that 20-HETE may play an important role in mediating ET-1-dependent constriction in renal arterioles; however, interactions between ET-1 and CYP4A have not yet been studied in extra-renal vascular beds.

It has been reported that CYP enzymes have no effect on adrenergic vasoconstriction in renal arterioles; however, since the non-specific inhibitor 17-ODYA reduces both 20-HETE and EET formation, little specific information could be deduced from this investigation.[87] In mesenteric arteries, however, DDMS treatment reduces phenylephrine sensitivity in SHR and WKY animals.[66] Furthermore, co-incubation of the arteries with DDMS and 20-HETE prevented this change.[66] In agreement with earlier studies, the relative contribution of DDMS to vasoconstriction was higher in SHR than WKY rats.[66] These studies therefore suggest that CYP4A/20-HETE modulation of α_1-adrenergic vasoconstriction in mesenteric arteries is increased in SHR but is also important in normotensive animals.

5.3. 20-HETE-Dependent Vasoreactivity

Exogenous 20-HETE has primarily been reported to cause arterial vasoconstriction;[23] however, there are also some reports of exogenous 20-HETE (at the same concentrations) having a vasodilatory role.[91,102,103] These disparities are partly attributed to the cellular location of the 20-HETE formation, the size of the vessel being studied, and the animal species. This section will outline the effects of exogenous 20-HETE that have been reported thus far.

Treatment of cerebral arterioles with 20-HETE (1-100 nmol/L) causes vasoconstriction (and increased intracellular calcium) by inhibiting large conductance calcium-activated potassium (BK_{Ca}) channels[86] and by activating large conductance calcium channels.[89] Similarly, 20-HETE (1 nmol/L – 50 μmol/L) results in concentration-dependent constriction of small renal arterioles from Sprague-Dawley rats.[104-106] 20-HETE also constricts arcuate arteries from mongrel dogs[107] and porcine coronary arteries[108] at concentrations of 0.01-1 μmol/L. In rabbits, 20-HETE induced vasoconstriction in aortae as well as carotid, mesenteric and renal arteries.[109] Interestingly, carotid, mesenteric, and renal arteries were more sensitive to 20-HETE than were aortae (threshold for constriction = 10 nmol/L vs. 100 nmol/L) suggesting that 20-HETE plays a more important role in resistance arteries relative to conductance arteries.[109] In contrast, another study reported that 20-HETE (0.1 nmol/L – 1 μmol/L) only induced vasoconstriction in rabbit afferent arterioles that had been pre-constricted with norepinephrine.[110] The reason for the disparity between these studies in unclear. The mechanisms of the vasoconstrictor actions of endogenous 20-HETE will be discussed in section 5.4.

Recent studies have demonstrated vasodilation induced by 20-HETE in pulmonary arteries. In these arteries, application of 20-HETE (1 μmol/L)

activates eNOS to form NO resulting in vasodilation.[102,111] The authors hypothesize this may be due to the unique localization of CYP4A to the vascular endothelium in the lung.[102,112] In other tissues, CYP4A enzymes are expressed in the vascular smooth muscle cells.[89,113-115] Interestingly, 20-HETE (5 nmol/L – 10 μmol/L) has also been reported to cause vasodilation of mouse basilar[103] and bovine coronary[91] arteries. The mechanisms underlying 20-HETE-induced vasodilation as yet unclear but seem to be endothelium- and COX-dependent.[91,102,111]

5.4. 20-HETE-Dependent Cellular Signaling

Despite the contradictory actions of studies involving exogenous 20-HETE, endogenous 20-HETE acts intracellularly to cause constriction of the smooth muscle cell in which it was generated.[23] Although no specific 20-HETE receptor has been identified, there are numerous mechanisms through which 20-HETE has been reported to cause vasoconstriction. This section will summarize these findings, beginning with the liberation of AA from the plasma membrane.

In 1991, Kauser *et al.* noted that, similar to CYP inhibition, phospholipase A2 (PLA_2) inhibition reduced myogenic tone development in renal arcuate arteries from dogs.[84] In addition, vasoconstriction induced by several agonists is dependent upon phospholipase activation. Indeed, angiotensin II-dependent constriction is attenuated by PLA_2 inhibition in renal arteries from Sprague-Dawley rats.[116] Furthermore, stimulation of vascular smooth muscle cells with ET-1 activates both PLA_2[117] and PLC[118] while phenylephrine[119] and norepinephrine[120] can activate phospholipase D (PLD). As discussed in section 2, these phospholipases can act to cleave AA from the plasma membrane. Liberated AA then serves as a substrate for CYP4A hydroxylation reactions. The general outline for agonist-dependent 20-HETE generation in vascular smooth muscle cells is depicted in Figure 1.4. Subsequent to its formation, 20-HETE has been shown to have a variety of downstream effects.

It is likely that the relative contributions of these pathways are determined by vascular bed and species.

20-HETE (1 nmol/L – 0.1 μmol/L) has been reported to inhibit potassium channels in cerebral[86] and renal arterioles.[90,98] Specifically, in renal arterioles, 20-HETE inhibits BK_{Ca} channels.[90] BK_{Ca} channels allow the flux of potassium ions out of the cell thus promoting hyperpolarization and relaxation of vascular smooth muscle cells.[121] BK_{Ca} channels are activated by calcium sparks generated by ryanodine receptors on the sarcoplasmic reticulum and thus, the current of potassium ions that passes through them is referred to as the spontaneous transient outward current. The spontaneous transient outward current acts to maintain a more negative membrane potential and therefore to close voltage-gated calcium channels.[121] BK_{Ca} channels are also activated by the increased intracellular calcium and membrane depolarization that occurs at the beginning of vascular smooth muscle cell contraction.[121] Therefore, by inhibiting the potassium ion flux through BK_{Ca} channels, 20-HETE acts to promote cellular depolarization. In addition, 20-HETE (1 nmol/L – 0.1 μmol/L) can directly activate large-conductance voltage-gated calcium channels in cerebral arteries from cats.[89] These channels allow calcium influx when the plasma membrane becomes depolarized.[122] This finding also indicates that 20-HETE acts on membrane-bound ion channels to encourage membrane depolarization and therefore vasoconstriction. Furthermore, a study in porcine coronary arteries has indicated that vasoconstriction caused by 20-HETE (0.1-1 μmol/L) may be due, in part, to 20-HETE-dependent activation of Rho-kinase.[108] Rho-kinase acts to sensitize smooth muscle cells to vasoconstriction by phosphorylating myosin light chain phosphatase and thus reducing its ability to dephosphorylate myosin light chain (phosphorylation of myosin light chain is a critical step in vascular smooth muscle contraction).[123] The known mechanisms through which 20-HETE can cause vasoconstriction are depicted in Figure 1.5. The specific contributions of these pathways in the various vascular beds remain to be determined.

In addition to its vasoactive effects, 20-HETE (0.5 μmol/L) stimulates vascular smooth muscle cell proliferation by activating PLD (downstream of initial agonist-induced phospholipase activation)[113,114] or p38 mitogen-activated protein kinase.[124] Activation of this kinase also results in a positive feedback loop whereby 20-HETE acts to increase its own synthesis through a p38 mitogen-activated protein kinase-induced increase in PLA_2 activity. The role of 20-HETE-dependent cell proliferation may be of substantial importance in inflammatory diseases such as atherosclerosis.

20-HETE can be metabolized by numerous enzymatic processes including β-oxidation, esterification, binding to proteins, and (as mentioned above) metabolism by COX enzymes[23]. Furthermore, 20-HETE can be stored within the membrane phospholipid pools.[23] These reactions result in the formation of metabolites that are both less active (e.g. β-oxidation) or similarly active (e.g. metabolism by COX enzymes).[23] Since COX enzyme activity is profoundly affected by aging (see section 3.1), it may be important to investigate the effects of aging on these pathways and therefore on the activity of 20-HETE metabolites.

6. CYP4A and 20-HETE in Development & Disease

As has been described above, CYP4A production of 20-HETE plays an important role in vasoconstriction in numerous vascular beds of normotensive, healthy animals. In addition, this pathway contributes to the increased vasoreactivity noted in hypertensive SHR animals. This section will further characterize the physiological and pathophysiological actions of CYP4A and 20-HETE.

6.1. Development

In 1992, Omata *et al.* showed that 20-HETE production and ω-hydroxylase activity in renal cortical microsomes increased from birth until nine weeks of age in both SHR and WKY rats.[125] By 13 weeks of age,

however, ω-hydroxylase activity and 20-HETE production levels were reduced by two-thirds and reached a plateau that was maintained until the study was completed at 20 weeks of age.[125] Similar findings were reported in Dahl salt-sensitive rats.[126] Renal expression of the CYP4A isoforms found in rat tissue (CYP4A1, 4A2, 4A3 and 4A8) is also developmentally regulated. CYP4A1, CYP4A3, and CYP4A8 mRNA levels peak at three to five weeks of age and are subsequently reduced by ten weeks of age in WKY and SHR rats.[127] In contrast, these authors found that CYP4A2 mRNA levels increased from five to ten weeks of age and then plateaued until the study concluded at 14 weeks of age.[127] Expression of CYP4A3 and CYP4A8 in SHR kidneys was higher than that in WKY at three weeks of age but no differences in any of the isoforms were detected at later age points.[127] These data further support the hypothesis that increased CYP4A activity is important in the early generation of hypertension in the SHR. Furthermore, they suggest a role for CYP4A/20-HETE in the development of normotensive animals. However, the origin of the CYP expression and activity in these studies is unclear since the homogenates contained both renal arterioles and nephron components. The roles of vascular CYP4A in development and in aging past 20 weeks (early adulthood) remain to be determined. The role of vascular CYP4A in advanced age is the primary question addressed in this thesis (see section 6.3).

6.2. Hypertension

The role of the CYP4A/20-HETE pathway in hypertension is complicated because it has both pro- and anti-hypertensive properties within the kidney.[128] In addition to its vasoconstrictor actions on renal arterioles that can reduce renal filtration (discussed above), 20-HETE can act on various nephron segments to reduce sodium absorption. However, data regarding the extra-renal vasculature suggest that CYP4A production of 20-HETE profound pro-hypertensive effects in these arteries. This section will address the overall involvement of the CYP4A/20-HETE pathway in hypertension.

20-HETE inhibits the activity of the Na^+/K^+ATPase in the proximal convoluted tubule and the Na^+-K^+-$2Cl^-$ transport in the thick ascending loop of Henle.[129,130] Through these mechanisms, 20-HETE reduces sodium reabsorption, induces a natriuresis and, consequently, reduces blood pressure. Accordingly, induction of CYP4A expression with the anti-hyperlipidemic drug, fenofibrate, reduces blood pressure in stroke-prone SHR and Dahl salt-sensitive rats on a high salt diet.[131] In another study, young Sprague-Dawley rats were fed a high fat diet which resulted in increased blood pressure and reduced urine volumes accompanied by reduced expression of CYP4A1 and CYP4A8.[132] Similarly, treatment with another anti-hyperlipidemic drug, clofibrate, increases 20-HETE formation and CYP4A expression while normalizing blood pressure and urine volume in these animals.[132] These studies have highlighted the powerful anti-hypertensive actions of 20-HETE in the nephron.

In contrast, as mentioned in section 4.3, early studies regarding the induction of heme oxygenase suggested that inhibition of CYP activity reduces blood pressure in the SHR.[79,80] Later it was determined that specific inhibition of the ω-hydroxylation of AA reduces blood pressure in SHR animals.[133,134] Moreover, 20-HETE mediates the increased sensitivity to several vasoconstrictors in resistance arteries from SHR, animals.[66,100] In renal arteries, however, it has recently been shown that it is not an increase in 20-HETE synthesis, but rather a reduction in the production of 18- and 19-HETE by another CYP enzyme that results in increased arterial vasoconstrictor sensitivity.[24] Other studies have also reported that 19-HETE may play a vasodilatory role by acting as an endogenous physiological inhibitor of 20-HETE.[105,135] Altogether, these findings suggest the vasoconstrictor actions of 20-HETE may contribute to the development of hypertension in the SHR.

Recent studies with molecular genetic techniques have provided further data regarding the pro-hypertensive roles of CYP4A. In SHR animals,

intravenous injection of CYP4A1 antisense oligonucleotides reduced blood pressure and the sensitivity of mesenteric arteries to phenylephrine (normally increased in SHR relative to WKY animals).[136] Indeed, even in normotensive Sprague-Dawley rats, treatment with antisense CYP4A1 and/or CYP4A2 oliognucleiotides reduced blood pressure and renal arteriolar production of 20-HETE.[137] By contrast, intravenous delivery of CYP4A genes (with a CYP4A1 adeno-associated viral vector[138] or CYP4A2 adenovirus[139]) increased blood pressure in Sprague-Dawley rats. These studies involving systemic modulation of CYP4A activity have suggested that CYP4A has a net pro-hypertensive effect that may be due to CYP4A-dependent vasoconstriction in the periphery.

The role of the CYP4A/20-HETE pathway in humans is still unclear. 20-HETE-dependent natriuresis has been reported to be impaired in patients with salt-sensitive essential hypertension.[140,141] In addition, a variant of human CYP4A11 that codes for an enzyme that generates less 20-HETE is associated with essential hypertension.[142] Urinary 20-HETE excretion is positively correlated with hypertension in women and positively related to Body Mass Index and peripheral vascular dysfunction in both genders.[143] These data suggest that CYP-dependent production of 20-HETE may play an important role in the regulation of blood pressure in humans. Elucidation of the specific anti- and pro-hypertensive properties of 20-HETE will require future studies.

6.3. Aging

There have been no previous studies regarding advanced age and CYP4A expression; however, other studies have reported significant correlations between liver-derived CYP activity and aging.[144] Therefore, determining the role of aging on vascular CYP4A expression may be of substantial interest.

Increased oxidative stress in the vasculature is an important feature of aging. Through the generation of superoxide, CYP enzyme activity may

contribute to this stress (see section 4.1). Reactive oxygen species production in rat liver microsomes is both NADPH- and CYP-dependent.[145] Furthermore, in human microsomes from a human β-lymphoblastoid cell line, CYP1A1, CYP1A2, CYP2B6 and CYP3A4 catalyzed the formation of superoxide.[146] More recently it has been demonstrated that CYP enzymes in porcine coronary endothelial cells also produce significant amounts of reactive oxygen species.[147] Although these data suggest that uncoupling of CYP enzymes may contribute to oxidative stress in the vasculature, the effect of aging on CYP enzyme coupling is currently unknown. In addition, since NO can inhibit CYP4A activity,[148,149] reduced NO bioavailability in the aged vasculature (due to increased oxidative stress) may increase the importance of CYP4A modulation of vascular function in aging.

Given that aging is the primary risk factor for the development of cardiovascular diseases, and as our population ages, it is critical that we further elucidate the age-related mechanistic changes that contribute to vascular dysfunction. CYP4A-dependent production of 20-HETE is an important vasoactive pathway in resistance arteries from normotensive animals. Furthermore, this pathway mediates the increased peripheral vasoconstriction that occurs in animal models of hypertension. *We hypothesized that the contribution of CYP4A to vasoconstriction would be increased in the resistance vasculature from aged rats relative to young.*

7. Gender & Aging

In men, the risk of developing cardiovascular disease (CVD) increases in a relatively linear fashion with age.[150,151] However, hormonal status in aging women has a profound effect on the risk of development of CVD. Indeed, postmenopausal women are more likely to develop CVD than premenopausal women of the same age.[152,153] Furthermore, surgical ovariectomy or early menopause increases the risk of CVD.[154] Accordingly, hormone replacement therapy in postmenopausal women was expected to provide significant protection from CVD. However, numerous

epidemiological studies have demonstrated hormone replacement therapy to have no effect[155], or to be detrimental[156,157] in the prevention of CVD. These studies have largely discouraged the use of hormone replacement therapy to prevent or treat CVD in aging women. Therefore, further elucidation the menopause-induced mechanistic changes in the cardiovascular system and possible novel drug targets are of critical interest in our aging population.

Menopause in women,[158,159] and surgical removal of the ovaries in animals,[160-163] have been associated with reduced NO-dependent vasodilation and increased arterial responses to exogenous vasoconstrictors. As would have been anticipated, estrogen-replacement normalized vascular function in these studies.[159-162] Furthermore, estrogen replacement has been reported to reduce oxidative stress in ovariectomized rats.[164] *In vitro* treatment of resistance arteries with 17β-estradiol results in direct vasodilation that has been attributed to eNOS activation.[165] These data demonstrate that estrogen replacement can have positive effects on the vasculature, despite the epidemiological evidence that there is no improvement in the risk of CVD. Accordingly, it will be of great value to further understand the specific effects of estrogen on the aging vasculature.

Due to these profound effects of ovarian hormone deficiency on the vasculature, it is inadvisable to generalize the reported effects of aging on arterial function from studies regarding men or male animals to women or female animals. Thus, the effects of aging on CYP4A modulation of vasoconstriction in both male (Chapter 2) and female (Chapter 3) rats are reported in this thesis.

7.1. Gender/Hormonal Regulation of CYP4A

There have been very few studies regarding the effect of gender and/or hormonal status on CYP enzyme function. Moreover, male Cyp 4A14-knockout mice develop more severe spontaneous hypertension than age-matched females.[166] This hypertension develops as a result of an androgen-mediated increase in Cyp 4A12 expression and production of 20-HETE.[166]

Moreover, castration of male Cyp 4A14-knockout mice normalizes blood pressure while 5_{α}-dihydrodtestosterone (DHT) treatment increases blood pressure and Cyp 4A12 expression in females and castrated males.[166] Similarly, DHT treatment increases expression of CYP4A1 and CYP4A8, 20-HETE production, and blood pressure in male and female rats.[167] These data suggest that androgens may regulate the pro-hypertensive properties 20-HETE. Accordingly, the contribution of CYP4A-dependent 20-HETE production may be increased in males relative to females. The vast majority of the data regarding vascular CYP4A discussed above was collected in males. Thus, the role of the CYP4A/20-HETE pathway in females remains to be fully elucidated. However, there it has been reported that vascular 20-HETE production and CYP4A expression are increased in the early stages of pregnancy in rats.[168] These data provide support for the idea that 20-HETE may also be regulated by the hormonal milieu in female rats.

Due to the profound differences in the aging process between men and women, it is important to characterize the effects of aging on vascular function in both genders. In addition, CYP4A-dependent production of 20-HETE is increased by androgen treatment and altered by pregnancy suggesting that this pathway is hormonally regulated. *We hypothesized that CYP4A/20-HETE modulation of vasoconstriction would be increased in mesenteric arteries from aged male and female rats relative to their younger counterparts. Furthermore, we hypothesized that estrogen replacement in aged female rats would normalize the contribution of CYP4A/20-HETE to vasoconstriction.*

8. Thesis Overview

8.1. Thesis Aims

Using the aging Sprague-Dawley rat as a model, we aimed to:

- Investigate CYP4A modulation of vasoconstriction in aged male rats, including the potential involvement of the ET_A pathway (Chapter 2).
- Investigate CYP4A modulation of vasoconstriction in aged female rats, including the roles of ovarian hormone depletion and estrogen replacement (Chapter 3).
- Determine vascular expression of CYP4A (Chapter 4).

8.2. Thesis Structure

A version of Chapter 2 has been accepted with revisions for publication in the *Journal of Cardiovascular Pharmacology*. A version of Chapter 3 is in preparation for submission to the *Journal of Vascular Research*. In accordance with the paper-based thesis format outlined by the Faculty of Graduate Studies and Research, Chapters 2 and 3 have brief introductions summarizing the relevant aspects of the more detailed background provided in this introduction. The data presented are discussed individually in the chapters and the overall concepts are addressed in the generalized discussion in Chapter 4. Similarly, the data regarding vascular CYP expression in Chapter 4 are accompanied by a brief introduction and discussion.

9. Figures

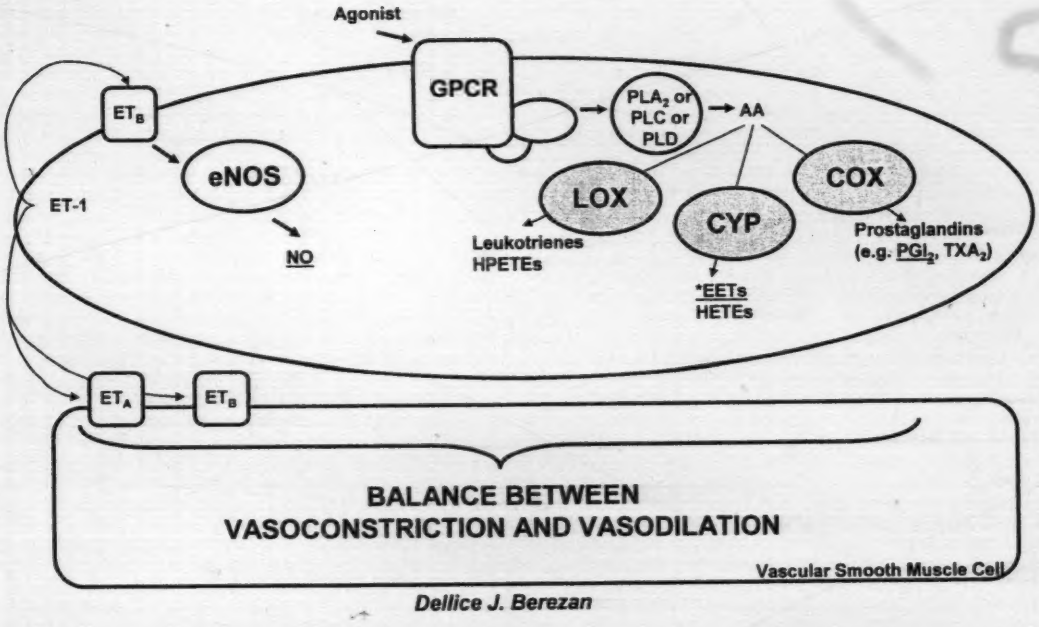

Figure 1.1 Endothelial modulation of vasoconstriction and vasodilation.

The schematic depicts endothelial modulation of the balance between vasodilation and vasoconstriction in the underlying vascular smooth muscle. Agonist-induced activation of a G-protein coupled receptor (GPCR) results in the activation of phospholipases (PLA_2/PLC/PLC) to result in the generation of free arachidonic acid (AA). AA is metabolized by three families of enzymes: (1) lipoxygenase (LOX) enzymes that generate leukotrienes and hydroperoxyeicosatetraenoic acids, (2) cyclooxygenase (COX) enzymes that generate prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), and (3) cytochrome P450 (CYP) enzymes that generate epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs). The three sources of endothelium-dependent dilation are underlined including nitric oxide (NO; formed by endothelial nitric oxide synthase [eNOS]), PGI_2, and EETs as a potential member of the group of factors referred to as the endothelium-derived hyperpolarizing factor. Finally, endothelin-1 (ET-1) can act on its endothelial (ET_B) or vascular smooth muscle (ET_A and ET_B) receptors to have profound effects on vascular tone.

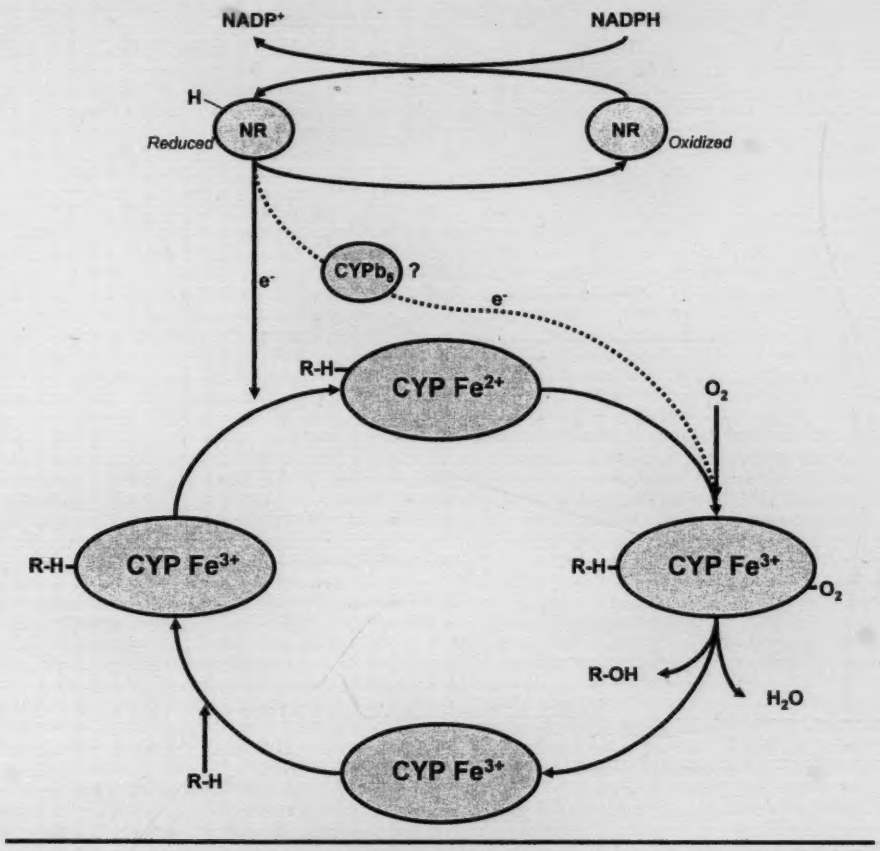

Figure 1.2 CYP4A enzyme-dependent oxidation.

Ferric CYP (Fe^{3+}) enzymes bind the substrate (R-H) to form a ferrous CYP-RH complex. This complex is reduced to form a ferrous ($Fe2^{+}$) CYP-RH complex by an electron from the NADPH-cytochrome P450 reductase (NR) reaction. A second electron and the addition of molecular oxygen (O_2) result in the re-generation of the ferrous enzyme complexed with the R-H and O_2 that quickly results in the formation of the oxidized substrate (R-OH) and water (H_2O). In the NR reaction, reduced nicotinamide adenine dinucleotide phosphate (NADPH) reacts with NR to form oxidized nicotinamide adenine dinucleotide phosphate ($NADP^{+}$) and reduced NR. Reduced NR then donates an electron to the ferrous CYP complex. The source of the second electron may also be the NR reaction; however cytochrome b5 ($Cypb_5$) may mediate this process. *Adapted from Correia (2001)[68]. Figure 4-3: Cytochrome P450 cycle in drug oxidations, pp. 53.*

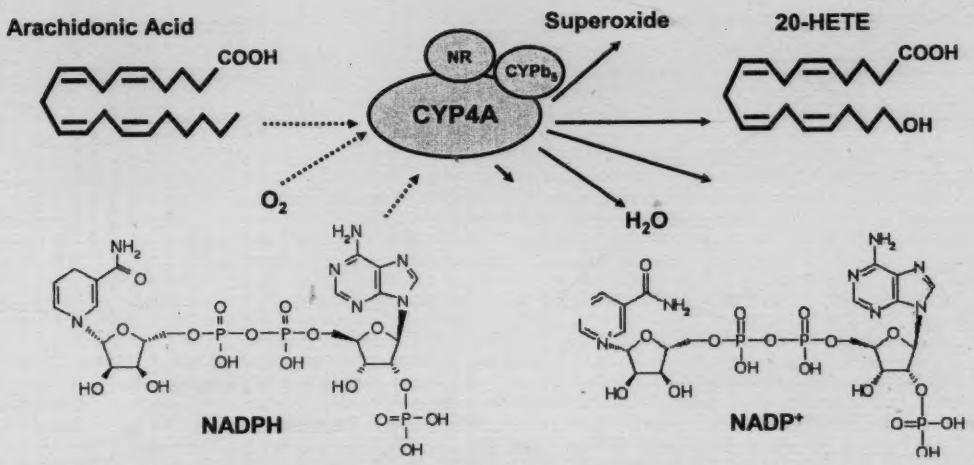

Figure 1.3 CYP4A-dependent ω-hydroxylation of arachidonic acid to form 20-HETE in vascular smooth muscle.

Arachidonic acid, molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) take part in the NADPH-cytochrome P450 reductase (NR)- and cytochrome b_5 ($Cypb_5$)-dependent production of 20-HETE, water and oxidized nicotinamide adenine dinucleotide phosphate (NADP+) by CYP4A enzymes. Superoxide can also be generated by this reaction. *Adapted from www.genome.jp; August 15, 2007.*

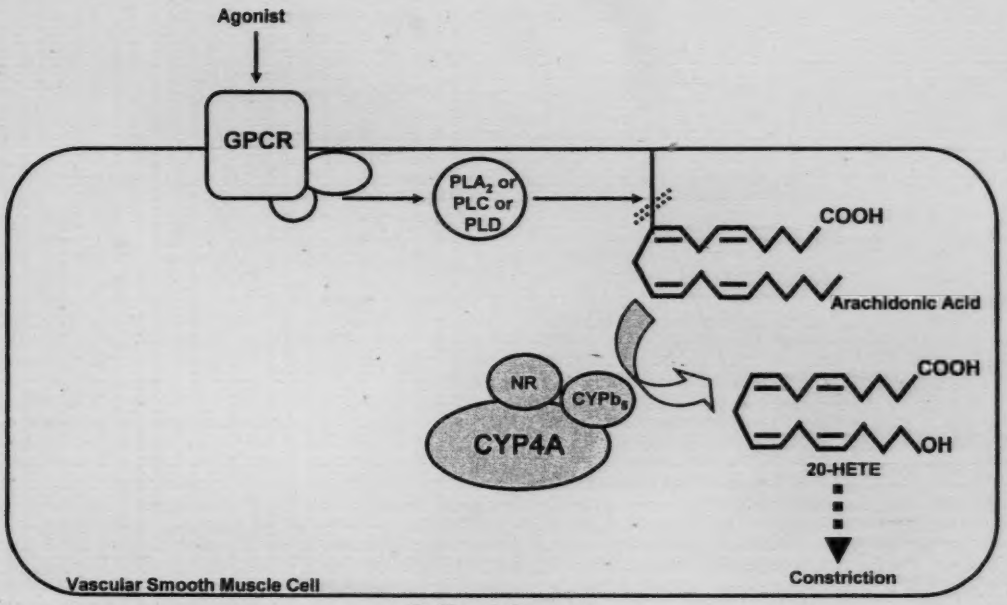

Figure 1.4 Potential mechanisms for agonist-induced activation of CYP4A-dependent 20-HETE formation in vascular smooth muscle.

Agonist binding to G-protein coupled receptors (GPCRs) may result in the activation of phospholipases A_2, C or D (PLA_2/C/D). These phospholipases cleave arachidonic acid from the plasma membrane which may then serve as a substrate for NADPH-cytochrome P450 reductase (NR)- and cytochrome b_5 ($Cypb_5$)-dependent production of 20-HETE by CYP4A enzymes. Subsequently, 20-HETE can cause contraction of vascular smooth muscle cells.

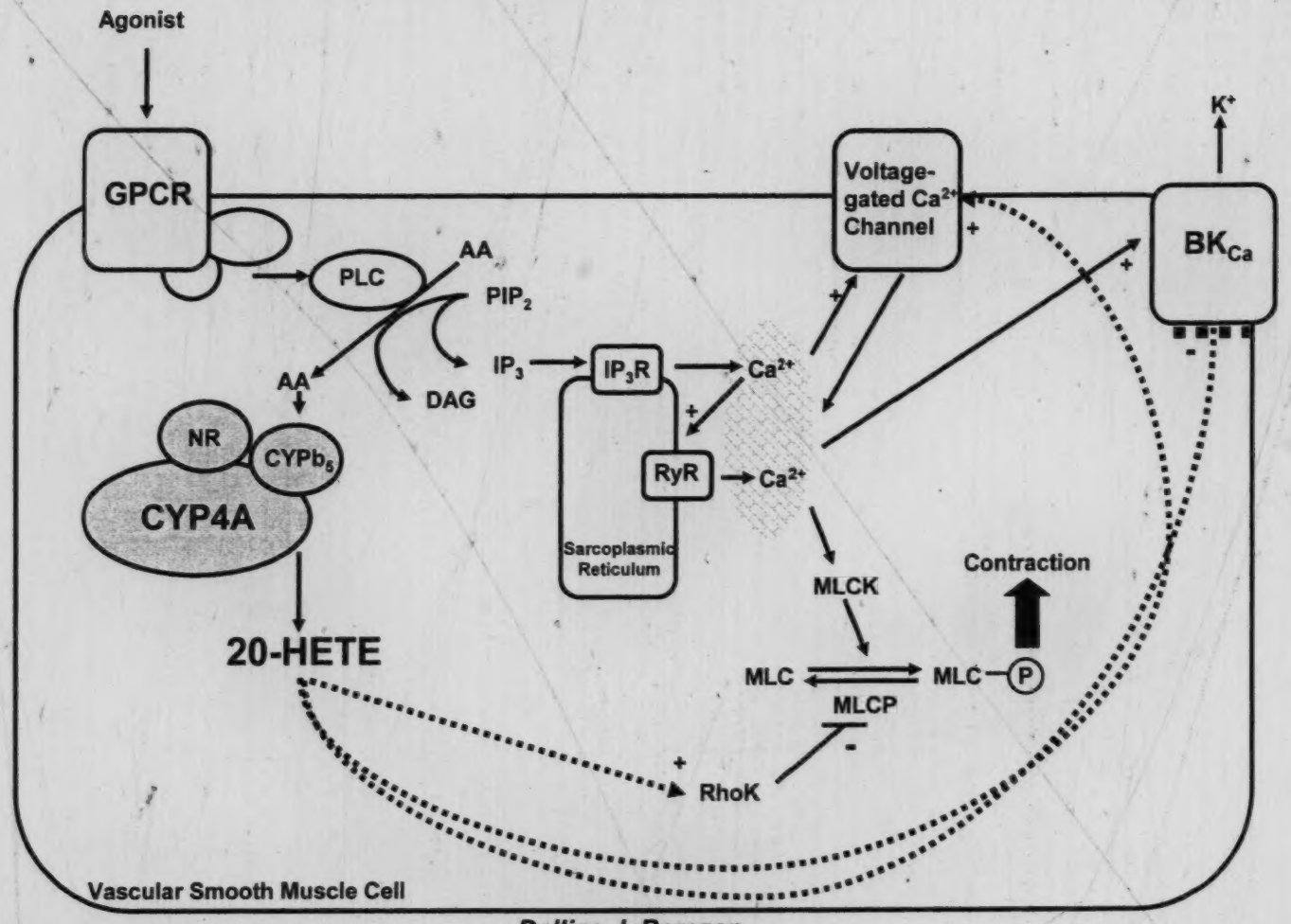

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Figure 1.5 Potential mechanisms for agonist-induced production of 20-HETE by CYP4A enzymes and reported mechanisms of action of 20-HETE.

Binding of agonists to G-protein coupled receptors (GPCRs) results in vasoconstriction through activation of phospholipase (PLC). PLC cleaves phosphatidylinositol bisphosphate (PIP_2) to form diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 binds to its receptors (IP_3Rs) on the sarcoplasmic reticulum to cause calcium (Ca^{2+}) release through IP_3R and ryanodine receptor (RyR) channels. Increased intracellular calcium activates both voltage-gated calcium channels that further increase intracellular calcium levels and, large-conductance calcium-activated potassium (BK_{Ca}) channels that act to restore membrane potential through potassium (K^+) efflux from the cell. Finally, increased intracellular calcium levels result in activation of myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chains (MLC) resulting in actin-myosin crossbridging and cellular contraction. MLC phosphatase (MLCP) dephosphorylates MLC and is negatively regulated by Rho-kinase (RhoK). Agonist-induced activation of phospholipases can also result in the liberation of arachidonic acid (AA) from the plasma membrane. AA can then serve as a substrate for NADPH-cytochrome P450 reductase (NR)- and cytochrome b_5 ($Cypb_5$)-dependent production of 20-HETE by CYP4A enzymes. Subsequently, 20-HETE acts to modulate contraction of vascular smooth muscle cells by activating voltage-gated Ca^{2+} channels and RhoK as well as inhibiting BK_{Ca} channels.

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CHAPTER II - AGING INCREASES CYTOCHROME P450 4A MODULATION OF α_1-ADRENERGIC VASOCONSTRICTION IN MESENTERIC ARTERIES FROM MALE RATS[1]

[1]A version of this chapter is under revision for *Journal of Cardiovascular Pharmacology*. Authors: D.J. Berezan, K. M. Dunn, J. R. Falck and S.T. Davidge.

- *K.M. Dunn quantified 20-HETE production in samples prepared by D.J. Berezan.*
- *J.R. Falck provided the CYP4A inhibitor DDMS as well as intellectual guidance.*

1. Introduction

Aging is associated with an increased risk of developing cardiovascular diseases such as heart failure,[1] atherosclerosis,[2] and hypertension.[3] Aging-related dysfunction of the peripheral vasculature may underlie the development of these pathologies. Moreover, aging reduces endothelium-dependent vasodilation,[4] and results in abnormal responses to vasoconstrictor stimuli.[5-8] Ultimately, aging results in increased peripheral vascular resistance that is due, in part, to increased vasoconstrictor responsiveness in systemic resistance arteries.[9] With an aging population, it is important to understand mechanisms that contribute to aging-related vascular dysfunction in order to identify novel therapeutic targets for the prevention of cardiovascular disease.

As discussed in Chapter 1, CYP4A enzymes form the potent vasoconstrictor 20-HETE through ω-hydroxylation AA.[10] 20-HETE acts a second messenger by modulating vascular tone in the renal,[11] cerebral,[12] coronary,[13] and resistance circulation.[14] In mesenteric resistance arteries, inhibition of CYP4A reduces arterial sensitivity to various constrictors including angiotensin II,[15] intraluminal pressure (myogenic tone),[16] and the α_1-adrenergic receptor agonist phenylephrine.[17] 20-HETE production and its contribution to vasoconstriction are elevated in spontaneously hypertensive rats (SHR).[17] Moreover, intravenous administration of antisense CYP4A1 oligonucleotides reduced blood pressure and attenuated 20-HETE modulation of vasoconstriction in these animals.[18] Furthermore, treatment of normotensive rats with CYP4A genes (through a CYP4A1 adeno-associated viral vector[19] or CYP4A2 adenovirus[20]) significantly increased their blood pressure. These data suggest a potential role for CYP4A enzymes and 20-HETE in the enhanced vascular tone and reactivity in hypertension. Previous studies have also noted developmental changes in CYP4A enzyme expression[21] and 20-HETE production[22] up to 20 weeks of age; however, the effect of advanced age on CYP4A modulation of vascular function remains to be determined.

A well described mechanism for 20-HETE is its modulation of vasoconstriction induced by ET-1.[23-25] ET-1 is formed in endothelial cells and elicits potent and sustained vasoconstriction by activating its receptors, ET_A and ET_B, on vascular smooth muscle cells.[26,27] ET_A receptors, however, are believed to be the primary mediators of ET-1-dependent constriction.[28-30] ET-1 binding of ET_A receptors results in PLA_2 activation and, subsequently, liberation of AA from the plasma membrane.[31] In renal arterioles, this leads to 20-HETE formation by CYP4A enzymes and subsequent vasoconstriction.[23-25] The involvement of this pathway in extra-renal resistance arteries remains to be determined. In aging, plasma ET-1 concentrations are elevated;[32,33] although, vascular ET-1 expression may be elevated[34] or reduced[35] in aging depending on vascular bed and species. Furthermore, ET_A gene expression was unaffected by aging in rat skeletal muscle arterioles.[36] Accordingly, the precise roles of the ET-1/ET_A and CYP4A pathways in resistance arteries during aging require further investigation.

We hypothesized that aging would increase the contribution of CYP4A to vasoconstriction in resistance-sized mesenteric arteries. Furthermore, we investigated the function of the ET_A receptor in aging as well as the potential role for CYP4A activity in ET-1-dependent vasoconstriction.

2. Materials and Methods

2.1. Chemical Reagents

All reagents were obtained from Sigma-Aldrich, St Louis, MO unless otherwise stated.

2.2. Animal Model

All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. Young (3 months) and aged (14 months) male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). The rats had *ad libitum* access to food and water. Young

rats were euthanized at 3-4 months of age while the aged rats were euthanized at 17-18 months of age. The rats were anaesthetized to surgical plane in the presence of 0.5 mL isoflurane (Halocarbon Laboratories, River Edge, NJ) and exsanguinated. A small section of the mesenteric arcade 5-10 cm distal to the pylorus was excised, and immediately placed in ice-cold *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]-buffered physiological saline solution (HEPES-PSS; pH 7.4; 142 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH_2PO_4, 1.17 mmol/L $MgSO_4$, 1.56 mmol/L $CaCl_2$, 10 mmol/L HEPES, 5.5 mmol/L glucose).

2.3. Pressure Myograph System

Arterial Preparation

In ice-cold HEPES-PSS, small (230-330 μm) mesenteric arteries were cleaned of all adipose and connective tissue. They were subsequently mounted onto a two chamber pressure myograph system (Living Systems Inc., Burlington, VT) containing ice-cold HEPES-PSS. The proximal end of each arterial segment was tied on to one glass cannula and any remaining blood in the artery was gently flushed out with HEPES-PSS. The distal end was then tied on to the second cannula and the artery was pressurized to 60 mmHg. Once the arteries were mounted, the buffer was replaced with HEPES-PSS that had been warmed to 37 °C. The temperature within the bath was continuously monitored and maintained at 37 °C (thermistor sensor and heating plate, Living Systems Inc.).

Equilibration

Once the vessels were warmed to 37 °C, they were allowed to equilibrate for a total of 50 minutes prior to experimentation. During this time, the HEPES-PSS was replaced every 10 minutes. As part of the equilibration protocol, the arteries were exposed to mildly increased intraluminal pressure for 10 minutes. Therefore, the protocol was as follows: 20 minute equilibration at 60 mmHg, 10 minutes at 80 mmHg, and 20 minutes at 60

mmHg. Pressures of 60-80 mmHg are considered to be physiological in this arterial bed.[37] Vessel diameter was recorded following equilibration using a video camera and video dimension analyser (Living Systems Inc.). There was no difference in size between arteries from young and aged rats (276 ± 10 μm [n = 7] vs. 300 ± 11 μm [n = 8], respectively). Throughout this thesis n will refer to the number of experimental animals.

2.4. Pressure Myograph Protocols

To determine the roles of CYP4A and ET_A in vasoconstriction, arteries were incubated in the absence or presence of the CYP4A inhibitor N-methylsolfonyl-12,12-dibromododec-11-enamide ([DDMS]; 10 μmol/L[2]; Dr. J. R. Falck, Dallas, TX) and/or the ET_A inhibitor BQ123 (1 μmol/L) for 20 minutes. Subsequently, the arteries were exposed to increasing concentrations of the α_1-adrenergic receptor agonist phenylephrine (5 nmol/L – 12 μmol/L) or ET-1 (0.1 – 50 nmol/L)[3]. Diameters were recorded two minutes after administration of each concentration of the vasoconstrictor.

Four arteries were assessed from each experimental animal. Only one concentration-dependent vasoconstriction experiment was conducted on each vessel. Thus, two sets of two vessels were mounted each day. Arteries were stored in ice-cold HEPES-PSS until the time of experimentation. The protocol was randomized such that the drugs applied to the first and second sets of vessels were rotated. Furthermore, time-control experiments displayed no difference between the vasoconstriction responses of the first and second sets of vessels.

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[2] DDMS selectively inhibits 20-HETE formation by CYP4A enzymes with an IC_{50} of 2 μmol/L.[38] At concentrations higher than 20 μmol/L DDMS, can also competitively inhibit the formation of epoxyeicosatrienoic acids by other CYP enzymes.[38] Thus, we used 10 μmol/L DDMS in this study. This dose has been used in several other investigations regarding the role of CYP4A production of 20-HETE in the vasculature and has been shown to be specific to inhibition of 20-HETE[39].[39,40]

[3] A synthetic peptide containing the sequence for human ET-1.

Data Analysis

Since all vessels reached maximal constriction (i.e. complete luminal occlusion) at the highest concentrations of phenylephrine or ET-1, data are represented as percent of maximal constriction. Furthermore, the concentration of vasoconstrictor that elicited 50% of maximal constriction (EC_{50}) was calculated for statistical analysis.

2.5. CYP4A Production of 20-HETE

Arterial Preparation

Separate experiments were conducted to determine the relative capacity of mesenteric arteries from young and aged rats to produce 20-HETE. Following euthanasia of the animal as described above, the entire mesenteric arcade was excised and placed in ice-cold HEPES-PSS. The arteries were cleaned of adipose and connective tissue and weighed. All of the arteries were collected to provide sufficient tissue volume for quantification 20-HETE. Subsequently, the arteries were incubated in a phosphate buffer containing: 40 µmol/L AA (Biomol International, LP, Plymouth, PA), 1 mmol/L NADPH, 2 µmol/L indomethacin (Biomol International, LP), 10 mmol/L $MgCl_2$, and 2 µmol/L ethylenediamine-tetraacetic acid at 37 °C for 90 minutes. 100% oxygen was streamed over the top of the reaction vials. These conditions were specifically designed to optimize 20-HETE production by K.M. Dunn in the laboratory of Dr. R. J. Roman at the Medical College of Wisconsin (Milwaukee, WI). It is important to note that there was an excess of AA substrate in these preparations.

20-HETE Extraction and Quantification

After 90 minutes, the reaction was stopped by acidification with 1 mol/L formic acid. Subsequently, the arteries were homogenized manually with a glass homogenizer at room temperature. Three mL of ethyl acetate were added to each sample and centrifuged at 900 *g* for three minutes. The organic phase (containing 20-HETE) was extracted by glass pipette. This was repeated once

more with ethyl acetate, and once with deionized distilled water. The final organic product was dried under nitrogen gas, frozen at -80 °C and shipped to K.M. Dunn (Medical College of Wisconsin, Milwaukee, WI) on dry ice. Upon arrival, the samples were reconstituted in 25 µL methanol and 25 µL of deionized distilled water. Five ng of the internal standard 20-hydroxyeicosa6(*Z*),15(*Z*)-dienoic acid (WIT-002) was added to each sample. 20-HETE production was then quantified by liquid chromatography-mass spectrometry as previously described.[41] The amount of 20-HETE produced is reported in ng of 20-HETE per g of wet tissue weight (all dissected arteries) per minute.

2.6. Statistics & Data Analysis

Data are expressed as mean values ± standard error of the mean. The significance of difference in mean values between and within groups were determined by t-test or 1- or 2-way analysis of variance (ANOVA) with Tukey's *post hoc* test, where appropriate. A p value < 0.05. was considered to be significant.

3. Results

Neither phenylephrine- nor ET-1-induced vasoconstriction was significantly altered by aging; however, the contribution of CYP4A to vasoconstriction was age-dependent. In arteries from young rats, phenylephrine-induced vasoconstriction was not affected by DDMS (EC_{50}: Control = 0.65 ± 0.06 µmol/L vs. DDMS = 0.73 ± 0.15 µmol/L; Figure 2.1). However, in agreement with our hypothesis, administration of DDMS significantly reduced phenylephrine sensitivity in arteries from aged rats (EC_{50}: Control = 0.49 ± 0.04 µmol/L vs. DDMS = 0.89 ± 0.08 µmol/L; $p < 0.05$; Figure 2.1). In contrast, vasoconstriction with exogenous ET-1 was not altered by CYP4A inhibition in arteries from either young or aged rats (Figure 2.2).

To investigate possible aging-related interactions between CYP4A activity and endogenous ET-1 signalling, arteries were constricted with phenylephrine in the absence or presence of BQ123 alone or in combination with DDMS. In young rats, phenylephrine-induced constriction was not affected by BQ123 with or without DDMS (Figure 2.3 A & B). In aged rats, however, phenylephrine sensitivity was significantly reduced by treatment with BQ123 alone or in combination with DDMS (EC_{50}: Control = 0.49 ± 0.04 μmol/L, BQ123 = 0.69 ± 0.06 μmol/L, DDMS & BQ123 = 0.88 ± 0.07 μmol/L; ANOVA $p<0.001$; Figure 2.3 C & D). Moreover, the increase in EC_{50} due to incubation with BQ123 and/or DDMS was not different between groups ($p = 0.259$).

Finally, mesenteric arteries from young rats produced more 20-HETE than arteries from aged rats (0.67 ± 0.13 vs. 0.22 ± 0.04 ng 20-HETE/g of tissue/min, respectively; Figure 2.4).

4. Discussion

This is the first report of increased CYP4A modulation of vasoconstriction in advanced age. Earlier studies have reported developmental increases in 20-HETE formation and CYP4A gene expression in young rats.[21,22] However, by 13 weeks of age (early adulthood), 20-HETE and CYP4A levels had reached a plateau.[21,22] By studying rats at 17 to 18 months of age, our investigation has elucidated a novel mechanism that may underlie the progression of aging-induced alterations in vascular function.

Although our data indicate that CYP4A modulation of α_1-adrenergic constriction is increased in aging, the underlying mechanisms remain to be fully elucidated. α_1-Adrenergic receptors can directly activate PLD to cause AA release.[42] Thus, their activation may increase substrate availability for CYP4A enzymes to generate 20-HETE. 20-HETE is the primary vasoconstrictor synthesized by CYP4A in vascular smooth muscle cells.[43] It is believed to act intracellularly as a second messenger to promote calcium influx and vasoconstriction through depolarization of smooth

muscle cells by blocking BK_{Ca} channels[44] and increasing the open state probability of voltage-gated calcium channels.[45] Thus, there are many sites at which aging may cause alterations in α_1-adrenergic receptor signalling through CYP4A. For instance, vascular BK_{Ca} channel expression is reduced in aging;[46] therefore, it is possible that CYP4A production of 20-HETE in aged rats causes a more complete blockade of these channels resulting in increased CYP4A-dependency of α_1-adrenergic constriction. However, further study is required to determine the precise effects of aging on these pathways.

CYP4A modulation of phenylephrine-induced vasoconstriction of mesenteric arteries has been previously reported in normotensive, young adult WKY rats.[17] However, in agreement with another report,[16] CYP4A inhibition had no effect on phenylephrine-induced constriction in mesenteric arteries from young Sprague-Dawley rats in our study. In contrast, another investigation demonstrated that norepinephrine-dependent constriction was reduced by 30 μmol/L DDMS treatment in Sprague-Dawley rats.[47] This difference between this study and ours may be due to their utilization of a higher dose of DDMS that could have also inhibited the activity of other CYP enzymes. In addition, activation of adrenergic receptors other than α_1 by norepinephrine may have resulted in additional activation of CYP4A. Despite varying reports regarding the involvement of CYP4A in young animals, our data clearly demonstrate an increase in CYP4A modulation of α_1-adrenergic vasoconstriction in aged rats.

In this study there was no significant difference between the phenylephrine sensitivities of control arteries from young and aged rats. There was, however, a slight trend for increased phenylephrine sensitivity in arteries from aged rats. Previous studies have reported both increased and decreased α_1-adrenergic vasoconstrictor responses in aging.[5,36] Further study will be required to determine if there is a physiologically significant change in phenylephrine sensitivity in our model; however, we have clearly demonstrated that the contribution of CYP4A to phenylephrine-induced vasoconstriction is increased in aging.

Similar to the proposed adrenergic pathway above, ET_A receptors activate PLA_2[48] and C[49] in vascular smooth muscle cells. In renal arterioles, ET-1-induced liberation of AA results in the formation of 20-HETE which contributes to the vasoconstrictor response.[24] In contrast, in our study exogenous ET-1-dependent vasoconstriction was not affected by DDMS treatment in mesenteric arteries. This may be due to functional differences between the renal and mesenteric resistance vasculature. Additionally, the relative contributions of vasoactive pathways often depend upon the size of the vessel.[50] Renal arterioles have an approximate diameter of 15 μm and are constricted by about 40% (of maximal constriction) by 10 nmol/L ET-1[23] while this dose causes almost complete occlusion of the mesenteric arteries used in our study (initial diameter ~ 300 μm). Thus, CYP4A modulation of vascular function appears to be dependent upon vascular bed.

Similar to CYP4A inhibition, ET_A inhibition decreased phenylephrine sensitivity in aged, but not young, rats. This suggests that α_1-adrenergic vasoconstriction is partially dependent on ET_A activation in aged rats. Furthermore, co-inhibition of ET_A and CYP4A did not have an additive effect on phenylephrine sensitivity. Therefore, while exogenous ET-1 may cause an overwhelming vasoconstrictor stimulus, endogenously-mediated ET_A constriction may be acting through 20-HETE as a second messenger.

The finding that arteries from young animals were able to synthesize more 20-HETE *in vitro* than arteries from aged rats was unexpected. Since the increased CYP4A contribution to vasoconstriction is accompanied by increased *in vitro* 20-HETE production in SHR[43] and Dahl-salt-sensitive rats,[51] we had hypothesized that the arteries from aged rats would be capable of producing more 20-HETE. There are several possibilities that may explain this result. Firstly, the assay conditions do not reflect the environment *in vivo* given that all the necessary substrates were provided to arteries from both young and aged rats. For example, aging may increase basal or agonist-induced AA bioavailability allowing more 20-HETE to be generated in aged

animals than young *in vivo*. Indeed, aging increases AA levels in hearts from Wistar rats.[52] Secondly, the assay was conducted in the presence of 100% oxygen. It is possible that this oxygen, in addition to the increased oxidative stress present in aging vessels,[53] was toxic to the arteries from aged animals and inhibited their production of 20-HETE. Furthermore, since BK_{Ca} channel expression is reduced in aging,[46] the reduced amounts of 20-HETE in aging vessels may still have had a larger effect on smooth muscle cell depolarization by inhibiting more of the available channels. Finally, a recent study has reported that reduced synthesis of endogenous physiological antagonists of 20-HETE is responsible for the increased phenylephrine sensitivity in the SHR model.[54] Further studies are necessary to elucidate these complexities as they relate to aging.

In summary, CYP4A and ET_A modulation of α_1-adrenergic vasoconstriction are increased in aging rats. Furthermore, these two pathways may act in concert in the mesenteric vasculature. As our population ages, it is critical to understand the age-dependent mechanisms that underlie the development of vascular dysregulation. The CYP4A pathway may provide important targets for the prevention and management of cardiovascular disease in aging.

5. Figures

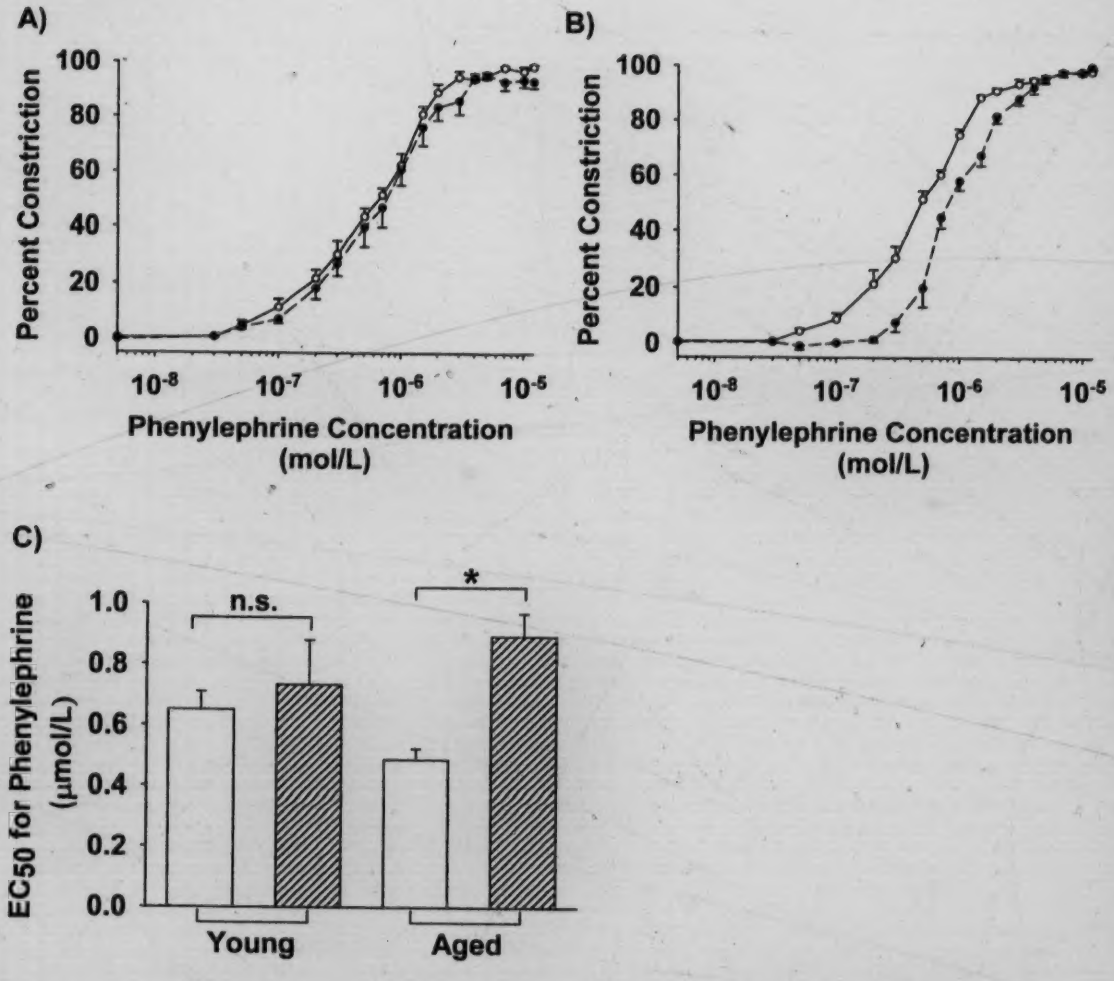

Figure 2.1 **Phenylephrine-dependent vasoconstriction in the absence or presence of CYP4A inhibition in arteries from young (3-4 months) and aged (17-18 months) rats.**

Phenylephrine-induced vasoconstriction in the absence (—o—) or presence (--•--) of the CYP4A inhibitor DDMS (10 µmol/L) in mesenteric arteries from **A.** young rats (n = 7) and **B.** aged rats (n = 7 - 8). **C.** Mean calculated EC_{50} values ± standard error of the mean for vasoconstriction in the absence (open bar) or presence (hatched bar) of DDMS (compared by 2-way ANOVA; Tukey's Test: * $p<0.05$ = aged control vs. aged DDMS).

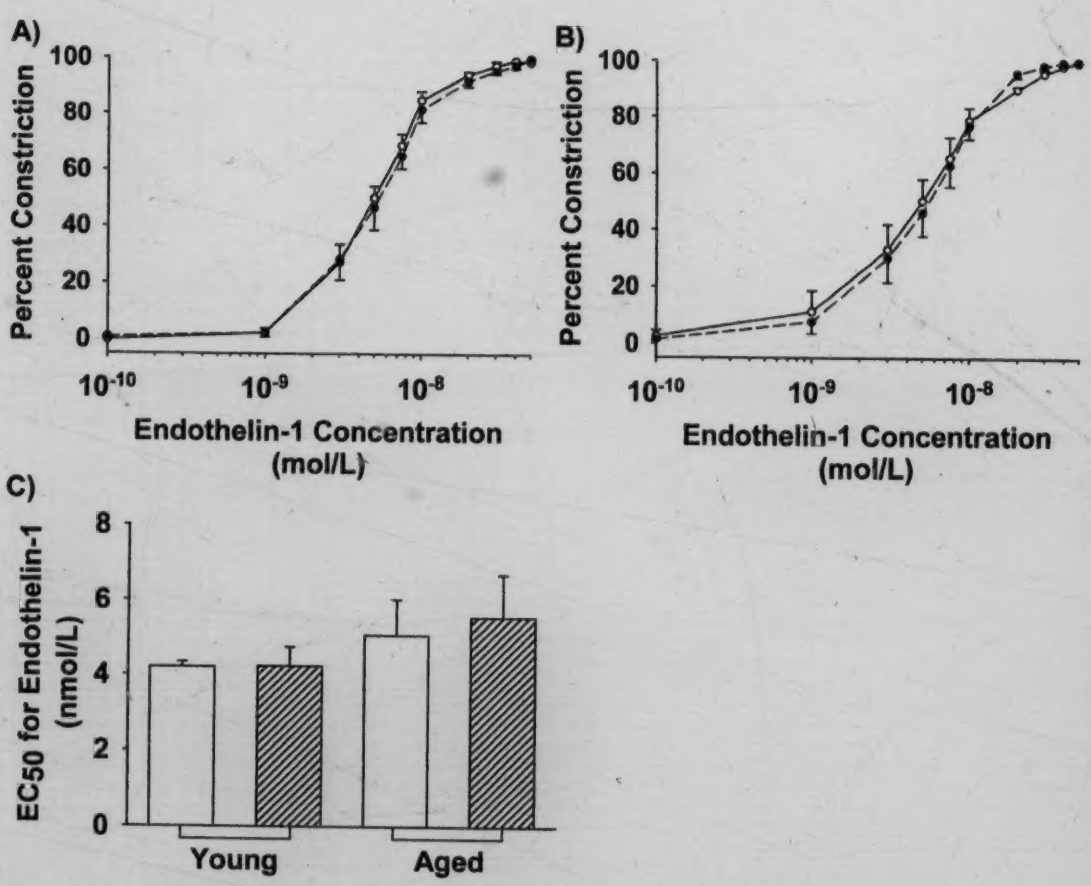

Figure 2.2 **ET-1-dependent vasoconstriction in the absence or presence of CYP4A inhibition in arteries from young (3-4 months) and aged (17-18 months) rats.**

Endothelin-1 (ET-1)-induced vasoconstriction in the absence (—o—) or presence (--●--) of the CYP4A inhibitor DDMS (10 μmol/L) in mesenteric arteries from **A.** young rats (n = 3) and **B.** aged rats (n = 5). **C.** Mean calculated EC_{50} values ± standard error of the mean for vasoconstriction in the absence (open bar) or presence (hatched bar) of DDMS (compared by 2-way ANOVA).

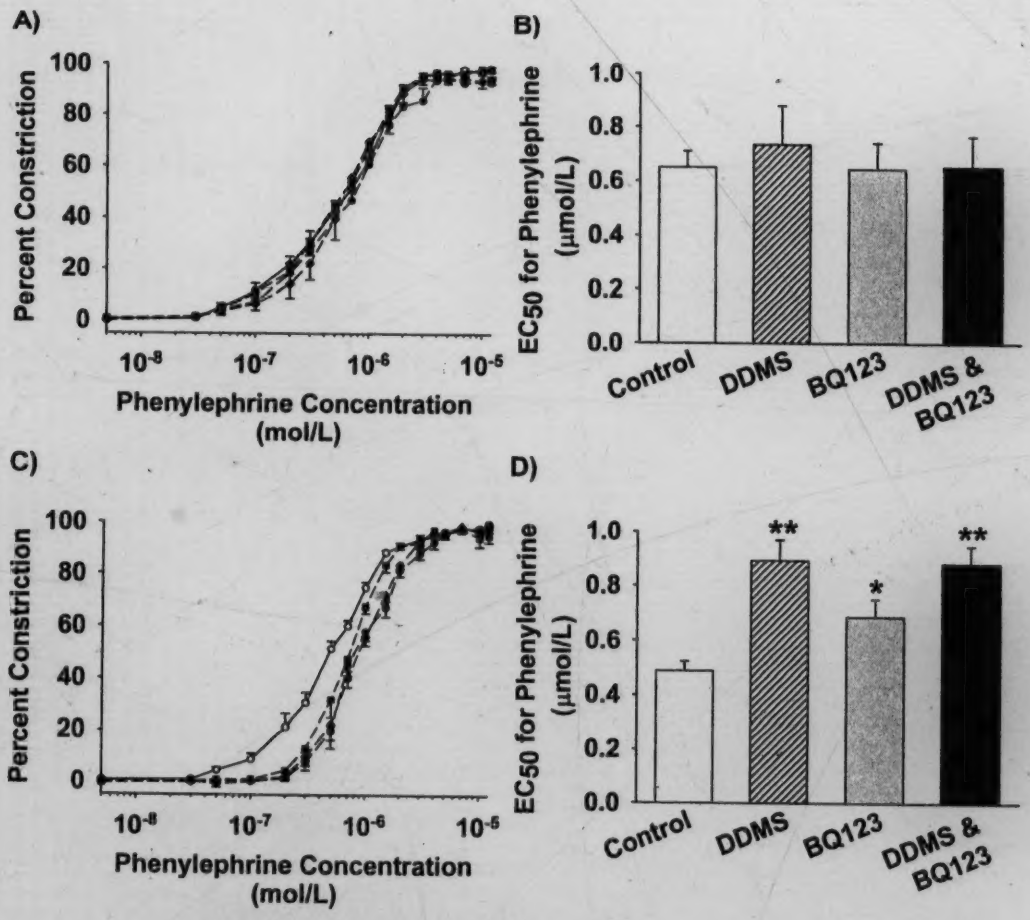

Figure 2.3 **Phenylephrine-dependent vasoconstriction in the absence or presence of CYP4A and/or ET_A inhibition in arteries from young (3-4 months) and aged (17-18 months) rats**

Phenylephrine-induced vasoconstriction in the absence (—o—) or presence of 10 µmol/L DDMS (--●--), 1 µmol/L BQ13 (--■--) or both DDMS and BQ123 (--♦--) in mesenteric arteries from **A.** young rats (n =6 - 7) and **C.** aged rats (n = 7 - 8). Mean calculated EC_{50} values ± the standard error of the mean for vasoconstriction in the absence (open bar) or presence of DDMS (hatched bar), BQ123 (grey bar) or both DDMS and BQ123 (black bar) in **B.** young and **D.** aged rats (compared by 1-way ANOVA; Tukey's Test: * $p < 0.05$ vs. control, **$p< 0.01$ vs. control).

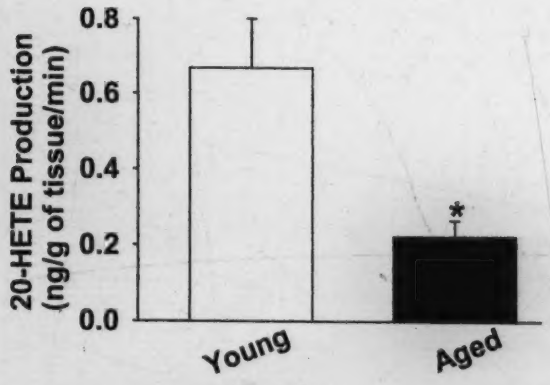

Figure 2.4 20-HETE production by arteries from young (3-4 months) and aged (17-18 months) rats.

In vitro 20-HETE production by mesenteric arterial arcades (in ng 20-HETE per g of wet tissue weight per minute) from young (n = 4; open bar) and aged (n = 8; black bar) rats (compared by t-test; *p = 0.032).

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CHAPTER III- OVARIECTOMY, BUT NOT ESTROGEN DEFICIENCY, INCREASES CYP4A MODULATION OF α_1-ADRENERGIC VASOCONSTRICTION IN AGING FEMALE RATS[4]

[4] A version of this chapter is under revision for *Journal of Vascular Research*. Authors: D.J. Berezan, Y. Xu, J. R. Falck and S.T. Davidge.

- *Y. Xu performed all the surgeries and subsequent animal care.*
- *J.R. Falck provided the CYP4A inhibitor DDMS as well as intellectual guidance.*

1. Introduction

Young women are less likely to develop CVD than men of the same age;[2] however, this protection is dramatically reduced at the time of menopause.[3] Moreover, menopause is associated with increased atherosclerotic disease[4] and hypertension.[2] Menopause-related peripheral vascular dysfunction may underlie these pathologies. Indeed, postmenopausal women have impaired endothelium-dependent vasodilation induced by both intraluminal flow[5] and acetylcholine,[6] as well as increased vasoconstriction responses.[7,8] These changes have been largely attributed to reduced ovarian hormone secretion, particularly estrogen.[9] Accordingly, hormone replacement therapy following menopause was expected to provide significant protection from CVD. However, clinical trials have found hormone replacement therapy to be beneficial,[10] have no effect,[11] or to be detrimental[12,13] in the prevention of CVD. As a result, it is necessary to further understand the mechanistic changes in the peripheral vasculature due to menopause in order to develop novel therapies for the prevention of CVD in aging women.

As previously discussed, CYP4A enzymes form the potent vasoconstrictor 20-HETE.[14] Studies in male animals have indicated that CYP4A production of 20-HETE partially mediates vasoconstriction caused by several agonists including the α_1-adrenergic receptor agonist phenylephrine.[15,16] 20-HETE also mediates pressure-induced myogenic constriction in cerebral,[17] renal,[18] and mesenteric[19] arteries from male rats. CYP4A modulation of vasoconstriction is elevated in peripheral arteries from spontaneously hypertensive rats[16] as well as hypertensive Dahl salt-sensitive rats.[20] Furthermore, antisense CYP4A mRNA reduces blood pressure in spontaneously hypertensive rats.[21] These data suggest that hypertension is accompanied by increased CYP4A activity in male rats. Recently, we have shown that aging also increases the contribution of CYP4A to α_1-adrenergic vasoconstriction in male rats (see Chapter 2). The role of CYP4A in aging females is currently unknown.

We hypothesize that ovarian deficiency in aging female rats will increase CYP4A modulation of α_1-adrenergic and myogenic vasoconstriction. We further hypothesize that these changes will be prevented by estrogen replacement.

2. Materials and Methods

2.1. Chemical Reagents

All reagents were obtained from Sigma-Aldrich, St Louis, MO unless otherwise stated.

2.2. Animal Model

All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Aged (15–16 months) female Sprague-Dawley rats (Charles River, Wilmington, MA) were assigned to be intact, ovariectomized (OVX), or ovariectomized and estrogen-replaced (OVX-E). Rats undergo reproductive senescence around 12 months of age;[22] however, they then enter a state of constant estrus[22] with consistent serum estrogen levels.[23] Therefore, to study aged females in an ovarian hormone-deficient state (i.e. menopause), we ovariectomized a subset of rats. At the time of surgery, a placebo or 17β-estradiol pellet (1.5 mg/pellet, 60-day release; Innovative Research of America, Sarasota, FL) was implanted subcutaneously. The rats were exposed to treatment for 4 weeks prior to experimentation. In our facility, intact female rats eat 4-5 pellets of food (Rat Chow 5001, LabDiet, Richmond, IN) per day. Therefore, all rats received this amount of food daily to account for the ovariectomy-induced hyperphagia that we have previously observed.[24]

On the experimental day, the rats were anaesthetized in the presence of 0.5 mL isoflurane (Halocarbon Laboratories, River Edge, NJ) and a midline

incision was made. The descending aorta was then severed for immediate blood collection resulting in exsanguination of the animal. A small section of the mesenteric arcade 5-10 cm distal to the pylorus was immediately excised and placed in ice-cold HEPES-PSS for vascular function studies. Effective ovariectomy or estrogen replacement was confirmed in three ways. Firstly, vaginal smears were taken to determine the stage of the estrus cycle (i.e. all intact and OVX-E rats should be in the estrus phase). Secondly, uteri were cleaned of connective tissue and their wet weight was recorded (estrogen has a trophic effect on uterine tissue[25]). Finally, serum 17β-estradiol levels were determined (see below).

2.3. Pressure Myograph System

Arteries were prepared and equilibrated as described in Chapter 2, section 2.3. Arterial diameter was not different between groups (Intact = 333 ± 10 μm, OVX = 328 ± 10 μm, OVX-E = 312 ± 8 μm; n = 9 in each group).

2.4. Pressure Myograph Protocols

To determine the role of CYP4A in modulating vasoconstriction, arteries were incubated in the absence or presence of 10 μmol/L DDMS (Dr. J.R. Falck, Dallas, TX). Each artery was exposed to increasing concentrations of phenylephrine (0.01-0.7 μmol/L) followed by a 30-minute washout period. The arteries were then re-incubated with either vehicle or DDMS (as appropriate) and subsequently exposed to increasing intraluminal pressure (0-140 mmHg). This was repeated in calcium-free PSS and with 100 μmol/L papaverine to determine the passive characteristics of the vessels. Diameters were recorded two minutes after phenylephrine administration and three minutes after each pressure step.

Data from phenylephrine-induced vasoconstriction experiments are reported as percent of maximal constriction as previously described. In this

study, the concentration of phenylephrine that elicited 20% of maximal constriction (EC_{20}) was calculated and used for statistical analysis. This point was chosen because of the distinct differences that occur at low concentrations of phenylephrine in arteries from female rats.

Data from pressure-induced constriction experiments are represented as percent myogenic constriction (active arterial diameter as a percentage of passive arterial diameter). Myogenic tone developed in approximately half of the arteries (Intact: 5 of 7; OVX: 5 of 9; OVX-E: 5 of 9). Accordingly, only these vessels were included in our analysis of myogenic constriction. This is consistent with previous studies of the mesenteric vasculature.[21]

2.5. Serum 17β-Estradiol

Blood samples were centrifuged at 2875 *g* for 10 minutes at 4 °C (Sorvall Instruments RC-3B/H-4000, Mandel Scientific, Guelph. ON). Serum was collected and frozen at -80 °C. Subsequently, 200 µL serum samples were sent to the Capital Health Diagnostics Services, Department of Laboratory Medicine and Pathology at the University of Alberta Hospital. Serum 17-β estradiol was then quantified using the Elcesys™ Systems 1010/2020/Modular Analytics E170 analyser and the Elecsys™ II Estradiol immunoassay kit (Roche Diagnostics, Laval, QE).

2.6. Statistics

Data were analyzed by t-test, 1- or 2-way analysis of variance (ANOVA) with Tukey's *post hoc* test where appropriate. All data are expressed as mean ± standard error of the mean and statistical significance was set at $p < 0.05$.

3. Results

3.1. Animal Model

As mentioned above, successful ovariectomy and estrogen replacement were verified in several ways. Firstly, intact and OVX-E rats were consistently in the estrus phase while OVX rats exhibited a diestrus-like phenotype (verified by vaginal smear). Furthermore, uterine weights from intact and OVX-E rats were higher than those from OVX animals (Intact: 0.75 ± 0.11 g, OVX-E: 0.89 ± 0.10 g vs. OVX: 0.29 ± 0.06 g, $p < 0.05$). Finally, there was no difference in serum 17-β estradiol levels between intact and OVX-E rats (59 ± 15 pg/mL [n = 6] and 64 ± 5 pg/mL [n = 3], respectively) while they were undetectable (< 30 pg/mL) in samples from four of five OVX rats. It was interesting to note that OVX rats gained 53 ± 6 g while OVX-E rats lost 35 ± 7 g between surgery and euthanasia. Furthermore, estrogen replacement appeared to have a negative effect on the rats' behaviour and appearance. Combined, these data may suggest that estrogen replacement in these aged animals was not favourable.

3.2. Assessment of Vasoconstriction

In accordance with other studies, ovariectomy increased phenylephrine sensitivity in arteries from aged female rats, but surprisingly, estrogen replacement further increased this sensitivity (EC_{20}: Intact = 0.49 ± 0.10 μmol/L vs. OVX = 0.21 ± 0.03 μmol/L vs. OVX-E = 0.12 ± 0.03 μmol/L; Figure 3.1). In agreement with our hypothesis, treatment with the CYP4A inhibitor DDMS reduced phenylephrine sensitivity in OVX (EC_{20}: Control = 0.21 ± 0.03 μmol/L vs. DDMS = 0.48 ± 0.07 μmol/L, $p < 0.05$; Figure 3.2, panels C and D) but not intact (Figure 3.2, panels A and B) rats. Interestingly, however, DDMS treatment in arteries from estrogen-replaced rats also reduced phenylephrine sensitivity (EC_{20}: Control 0.12 ± 0.03 μmol/L vs. DDMS 0.24 ± 0.05 μmol/L, $p < 0.05$; Figure 3.2, panels E and F). Moreover, the fold change from control with DDMS treatment was not different between

OVX and OVX-E animals (OVX: 1.41 ± 0.35 vs. OVX-E: 1.51 ± 0.68, $p = 0.112$).

We next determined the effects of ovariectomy and estrogen replacement on myogenic tone development. Arteries from OVX animals constricted more in response to pressure than those from intact animals at pressures below 100 mmHg (2-way ANOVA: $p < 0.05$; Figure 3.3, panel A). Estrogen replacement had no additional effect on vasoconstriction in this pressure range. Interestingly, the point at which the arteries underwent forced dilation was different among the groups (1-way ANOVA: $p < 0.05$; Figure 3.3, panel B). Arteries from intact rats continued to develop myogenic tone from 80 to 140 mmHg while there was very little change in percent constriction over this pressure range in arteries from OVX animals. Arteries from the OVX-E rats, however, demonstrated forced dilation between 80 and 140 mmHg. Finally, we tested the role of CYP4A in myogenic constriction. Contrary to our hypothesis, DDMS treatment did not alter arterial myogenic tone development in any of the groups (Figure 3.4). There were no differences in the passive properties of the arteries between groups (data not shown).

4. Discussion

Numerous studies have documented increased arterial responses to vasoconstrictors including phenylephrine following ovariectomy in young[26,27] and aged[28] rats. While estrogen replacement in young rats normalizes vascular function,[26,27] little is known about the effects of estrogen replacement on vasoconstriction in advanced age. In our study, estrogen replacement further increased phenylephrine sensitivity in aged female rats suggesting that, at this age, estrogen replacement may have detrimental effects on the vasculature. This finding may help to elucidate the mechanisms underlying recent conflicting epidemiological data. Indeed, the Women's Health Initiative found that hormone replacement with conjugated equine estrogens increased the overall risk of cardiovascular disease in an older population of women (50 - 79 years).[12] In contrast, the Nurses' Health Study

found that the relative risk of developing cardiovascular disease was significantly reduced in younger women (33 – 55 years) using estrogen therapy.[29] Our study has demonstrated that estrogen replacement in advanced age increased phenylephrine sensitivity which is due, in part, to CYP4A. Future studies may help to further understand aging-related mechanistic changes in vascular function that may lead to cardiovascular disease in aging females.

To our knowledge, this is the first study to demonstrate a role for CYP4A modulation of peripheral vasoconstriction in aging female rats. Our previous work has demonstrated that, in male rats, CYP4A modulation of α_1-adrenergic vasoconstriction in systemic arteries is increased in aged rats relative to young rats.[30] Therefore, CYP4A modulation of α_1-adrenergic constriction appears to be increased in aged, ovarian-depleted states. Moreover, in our aged female rats, estrogen replacement did not change the relative contribution of CYP4A to vasoconstriction. Previous studies have suggested that the role of vascular CYP4A is determined by the hormonal milieu and/or body mass. Indeed CYP4A activity is increased by androgen treatment in male rats[31] and differentially regulated during pregnancy in female rats.[32] Recent studies in humans have reported both positive[33] and negative[34] correlations between Body Mass Index and urinary 20-HETE excretion. In our study, the changes in body weight following ovariectomy in OVX (net gain) and OVX-E (net loss) animals would suggest that CYP4A modulation of vasoconstriction in mesenteric arteries is not correlated with body weight. Thus, body mass does not appear to be a primary determinant of CYP4A function in aged female rats. Since neither estrogen replacement nor body weight can explain the ovariectomy-induced increase in CYP4A modulation of α_1-adrenergic vasoconstriction that we observed, further study is required to elucidate the physiological changes that occur in these animals relative to CYP4A modulation of vasoconstriction.

In our aged rats, ovariectomy increased pressure-induced constrictor responses. Previous studies in skeletal,[35] cerebral,[36] and coronary[37] arteries from young rats have also reported increased myogenic constriction due to ovariectomy. However, in these

animals, estrogen replacement normalized myogenic constriction. In our aged rats, estrogen replacement did not normalize the enhanced pressure-induced constriction due to ovariectomy. Furthermore, estrogen replacement increased forced dilation. Indeed, the arteries from OVX-E rats began to dilate at pressures as low as 80 mmHg while arteries from intact and OVX rats did not undergo forced dilation even at 140 mmHg. Forced dilation resulting from high intravascular pressures has been reported in cerebral arteries;[38] in which it can lead to edema.[39] The effects of a reduced threshold for forced dilation in mesenteric arteries remain to be determined but may well be detrimental. Thus, estrogen replacement in aged rats appeared to result in further vascular dysfunction. It is important to note that little information is known regarding the pressure within the mesenteric vasculature in hypertensive states. Therefore, intraluminal pressures higher than normal (~ 80 mmHg) may be of limited physiological significance. Despite this limitation, our data clearly indicate that ovarian status has profound effects on pressure-induced alterations in arterial diameter in aged female rats. In addition, estrogen replacement had a negative effect on the appearance and behaviour of the animals. Thus, estrogen replacement in aged rats appeared to result in further vascular dysfunction as well as poorer condition of the animals.

Interestingly, CYP4A inhibition did not affect the development of myogenic tone in any of our aged rats. Development of myogenic constriction in mesenteric arteries from male Sprague-Dawley[19] and spontaneously hypertensive[21] rats was markedly reduced by pharmacological CYP4A inhibition and CYP4A antisense RNA treatment, respectively. Furthermore, CYP4A mediates renal[40] and cerebral[17] autoregulation in male rats. The role of CYP4A in modulation of myogenic tone has not been elucidated in female rats. Since CYP4A inhibition reduced phenylephrine sensitivity in our OVX and OVX-E animals, it appears that the CYP4A pathway is active in aged rats; however, it is not required for myogenic constriction in this vascular bed. Thus, these findings may suggest a specific aging-related increase in CYP4A modulation of agonist-dependent constriction. Phenylephrine binds to α_1-receptors which can activate PLD to cause AA release[41] and subsequent 20-HETE

formation by CYP4A enzymes. 20-HETE is believed to act intracellularly as a second messenger to cause smooth muscle cell depolarization by activation of voltage-gated calcium channels[42] or inhibition of BK_{Ca} channels.[43] Aging and hormonal status may affect this pathway at a variety of points. Thus, the mechanisms behind the increase contribution of CYP4A to α_1-adrenergic vasoconstriction in aged males and females remain to be determined. Nonetheless, our data indicate that CYP4A may be an important target for therapeutic intervention.

In summary, we have demonstrated that CYP4A modulation of α_1-dependent vasoconstriction is elevated in ovariectomized aged rats. Moreover, this pathway is not modulated by replacement with estrogen. In addition, our data illustrate that estrogen replacement in advanced age further increases α_1-adrenergic vasoconstriction and forced dilation. Further characterization of the roles of estrogen and CYP4A in advanced age may provide novel targets for the prevention of cardiovascular disease in aging females.

5. Figures

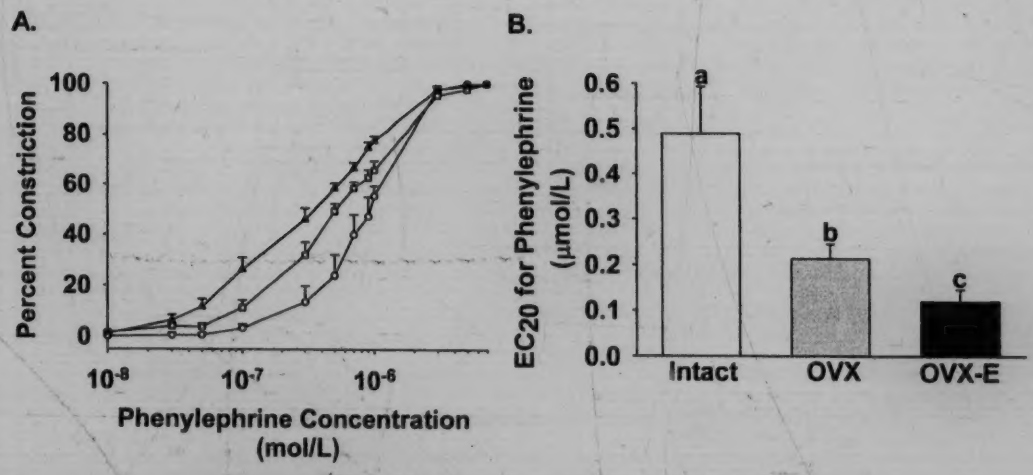

Figure 3.1 **Phenyelphrine-dependent vasoconstriction in arteries from aged intact, OVX and OVX-E rats.**

A. Phenylephrine-induced vasoconstriction in mesenteric arteries from intact (—○—, n=8), ovariectomized (OVX, —■—, n = 9), and ovariectomized and estrogen-replaced (OVX-E, —▲—, n = 9) rats. **B.** Bars represent the mean calculated EC_{20} values ± standard error of the mean for arteries from intact, OVX and OVX-E rats. Different letters denote significant differences among groups, $p < 0.05$.

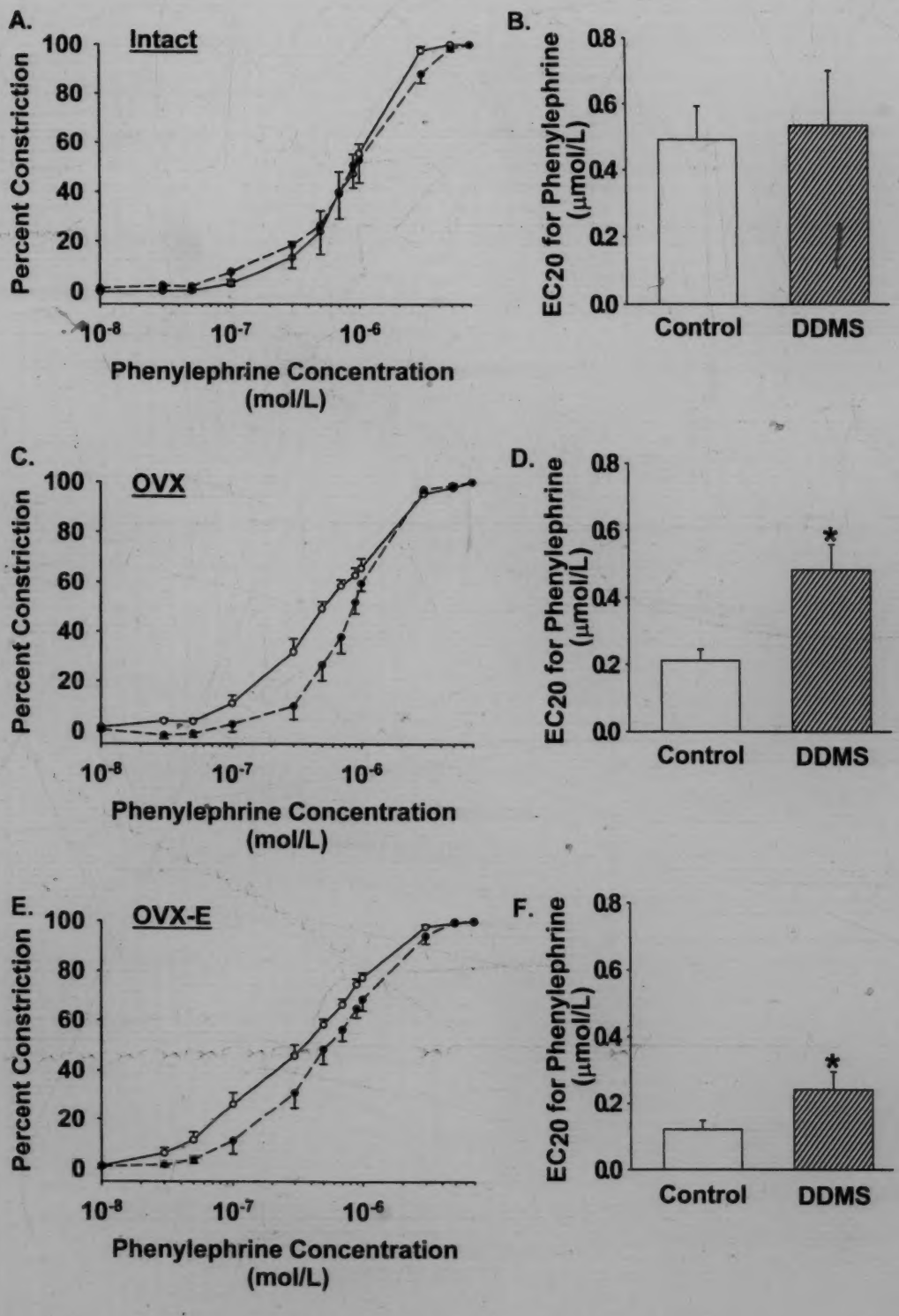
A.
Intact
Percent Constriction
100
80
60
40
20
0
10^{-8}
10^{-7}
10^{-6}
Phenylephrine Concentration
(mol/L)
B.
EC20 for Phenylephrine
(μmol/L)
0.8
0.6
0.4
0.2
0.0
Control
DDMS
C.
OVX
Percent Constriction
100
80
60
40
20
0
10^{-8}
10^{-7}
10^{-6}
Phenylephrine Concentration
(mol/L)
D.
EC20 for Phenylephrine
(μmol/L)
0.8
0.6
0.4
0.2
0.0
*
Control
DDMS
E.
OVX-E
Percent Constriction
100
80
60
40
20
0
10^{-8}
10^{-7}
10^{-6}
Phenylephrine Concentration
(mol/L)
F.
EC20 for Phenylephrine
(μmol/L)
0.8
0.6
0.4
0.2
0.0
*
Control
DDMS

Figure 3.2 Phenyelphrine-dependent vasoconstriction in the absence or presence of CYP4A inhibition in arteries from aged intact, OVX and OVX-E rats.

Phenylephrine-induced vasoconstriction in the absence (—○—) or presence (--●--) of the CYP4A inhibitor DDMS (10 μmol/L) in mesenteric arteries from **A.** intact (n = 8), **B.** ovariectomized (OVX, n = 9) and **C.** ovariectomized and estrogen-replaced (OVX-E, n = 9) rats. Bars represent mean calculated EC_{20} values ± standard error of the mean (*$p < 0.05$ vs. mean EC_{20} from vessels that did not receive DDMS).

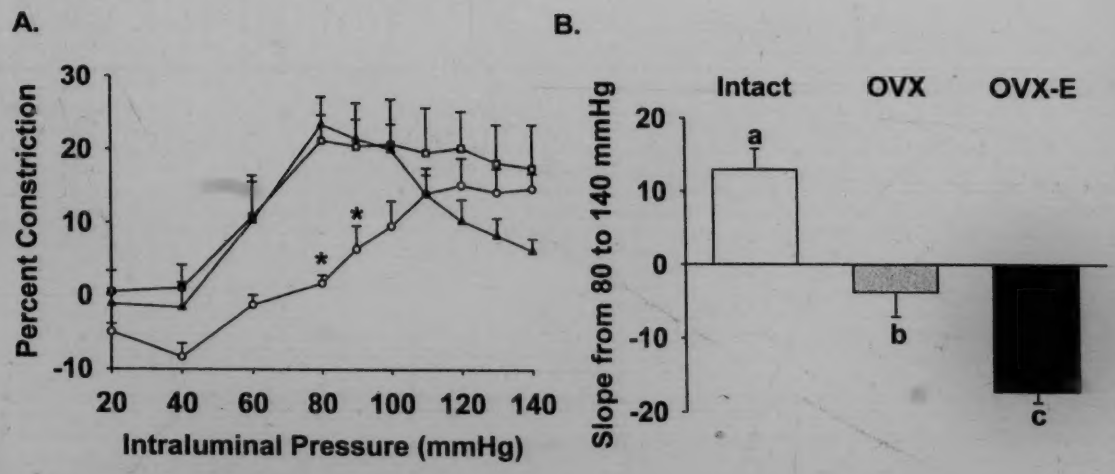

Figure 3.3 Pressure-dependent responses in arteries from aged intact, OVX and OVX-E rats.

A. Pressure-induced vasoreactivity in mesenteric arteries from intact (—○—, n = 5), ovariectomized (OVX, —■—, n = 5), and ovariectomized and estrogen-replaced (OVX-E, —▲—, n = 5) rats. **B.** Bars represent the mean slope from 80 to 140 mmHg ± the standard error of the mean for arteries from intact, OVX and OVX-E rats[5]. Different letters denote significant differences among groups, $p < 0.05$.

[5] This is reflective of the changes in arterial diameter over this pressure range. A positive slope indicates that arteries undergo constriction while a negative slope indicates an increase in arterial diameter.

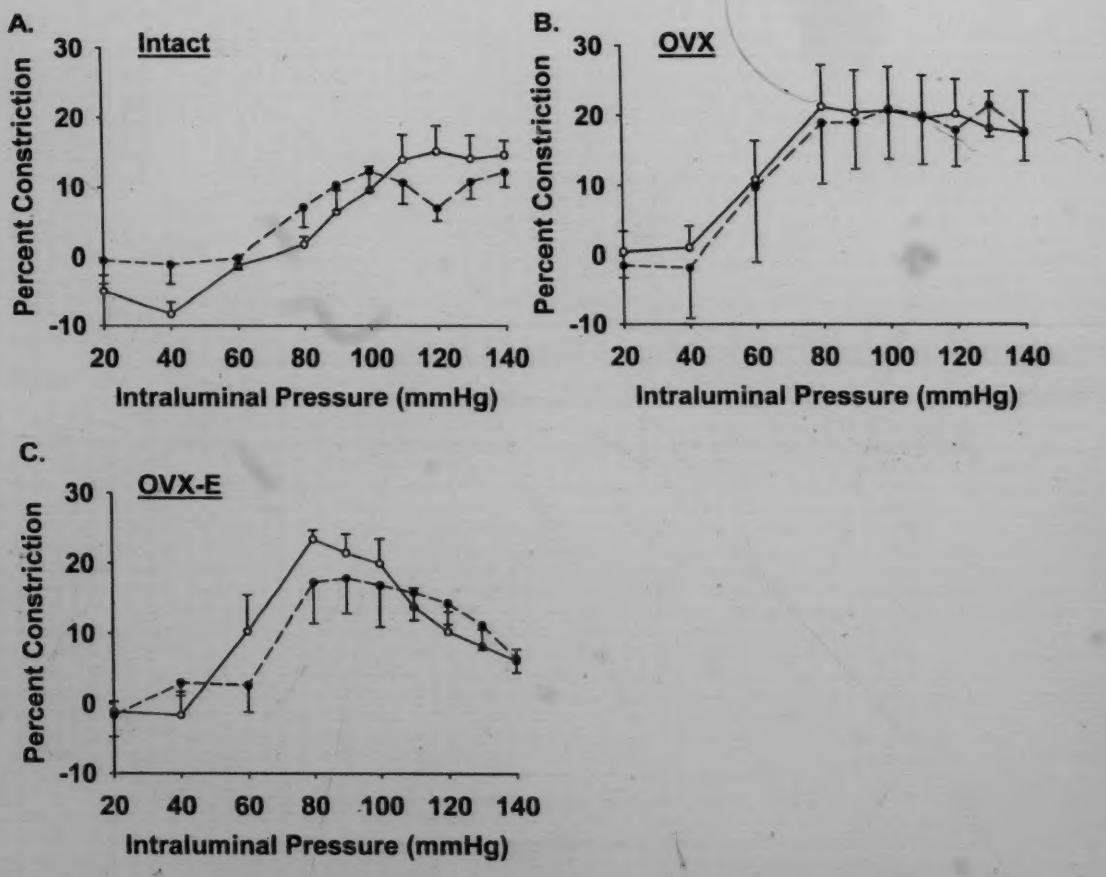

Figure 3.4 Pressure-dependent responses in the absence or presence of CYP4A inhibition in arteries from aged intact, OVX and OVX-E rats.

Pressure-induced vasoreactivity in the absence (—○—) or presence (--●--) of the CYP4A inhibitor DDMS (10 μmol/L) in mesenteric arteries from **A.** intact (n = 5-6), **B.** ovariectomized rats (OVX, n = 5) and **C.** ovariectomized and estrogen-replaced rats (OVX-E, n = 5).

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CHAPTER IV - VASCULAR EXPRESSION OF CYP4A

1. Introduction

Vascular CYP4A was first detected in cerebral arterioles from cats.[1] That study was conducted using an antibody raised against rat CYP4A2 that cross-reacts with the CYP4A1 and CYP4A3 isoforms.[1] Later studies demonstrated the expression of mRNA for four CYP isoforms in rat renal tissue, CYP4A1, CYP4A2, CYP4A3 and CYP4A8.[2-4] Renal genetic expression of CYP4A2 is increased during the development of hypertension in young SHR animals.[5] Furthermore, renal expression of all four isoforms are developmentally regulated in normotensive animals.[6] In mesenteric arteries, mRNA for CYP4A3 was detected in normotensive Sprague-Dawely rats while mRNA for all the isoforms were detected in animals exposed to a high fat diet.[7] These data suggest that expression of CYP enzymes can be regulated by a variety of environmental and genetic factors. The effect of aging on vascular CYP expression had not previously been studied; however, our functional data (Chapters 2 and 3) suggested that aging and ovarian status affected the functional activity of CYP4A in mesenteric arteries. Therefore, in accordance with our hypothesis, we expected that CYP4A mRNA and protein levels would be increased in mesenteric arteries from aged rats relative to young.

Due to the size of mesenteric arteries, they must all be collected for protein or mRNA quantification. As such, it is only possible to quantify one or the other for each animal. Our initial experiments using protein isolated from male rats indicated that the CYP4A antibody was problematic (see below). From that point onward, mesenteric arteries were only prepared for mRNA quantification. Thus, we have data regarding mRNA expression in arteries from male and female rats while protein expression was only examined in arteries from male rats.

2. Methods

2.1. Protein Quantification

Isolation of Mesenteric Arteries

Rats were euthanized as previously described. The entire mesentery was excised, rinsed in ice-cold HEPES-PSS and frozen in liquid nitrogen. The mesentery was then stored at -80 °C. Subsequently, the mesentery was thawed in ice-cold HEPES-PSS and each mesenteric artery was dissected free of fat and connective tissue and collected in 0.4 mL HEPES-PSS.

Protein Extraction

The samples were centrifuged at 11,180 *g* for two minutes at 4 °C. All of the HEPES-PSS was removed by glass pipette and the arteries were re-suspended in 100 µL of lysis buffer (100 mmol/L KCl, 0.5 mmol/L $ZnCl_2$, 10 mmol/L ethylenediamine-tetraacetic acid, 50 mmol/L Tris) with 10 µL Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO). The preparations were then sonicated on ice and centrifuged at 1006 *g* for five minutes at 4 °C. The supernatant was collected and stored at -80 °C

Bicinchoninic acid (BCA) assay

The quantity of protein in each sample was determined using the colorimetric BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). The principles of the assay are as follows. In an alkaline environment, protein interacts with Cu^{2+} to form a tetradentate-Cu^{1+} complex that is blue in colour. Subsequently, bicinchorinic acd (BCA) reacts with the newly formed Cu^{1+} ions to form a purple reaction product that absorbs radiation at 562 nm. Absorbance at this wavelength correlates linearly with the amount of protein in the sample.

Protocol

A series of dilutions (0-20 µg/µL protein) of bovine serum albumin (Sigma) were prepared to generate a standard curve for the BCA™ protein

assay (protein content vs. absorbance at 562 nm). Mesenteric artery samples were thawed on ice. 10 μL of each sample was diluted with 30 μL of the lysis buffer used for protein extraction. These dilutions, along with the bovine serum albumin standards, were pipetted into a 96-well plate. The BCA™ Assay reagents were added and the plate was incubated at 37 °C for 30 minutes. Absorbance at 562 nm was subsequently measured for each sample using a plate-reader (Molecular Devices UV Max, Sunnyvale, CA). The standard curve was used to determine protein concentrations for each mesenteric artery sample.

Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins within the samples by molecular weight. Briefly, samples were loaded into polyacrylamide gels and an electrical current forced the proteins to move across the gel due to their negative charge. The structure of the gel impedes the progress of the larger proteins resulting in separation of the proteins by molecular weight. SDS acts as detergent to denature secondary and tertiary protein structures allowing proteins to be separated based on amino acid chain length independent of protein conformation or folding.[8] Following separation of the proteins, they were transferred to a nitrocellulose membrane. Primary antibodies specific to the protein of interest were then able to bind to the appropriate epitope. A secondary antibody that fluoresces upon binding to the primary antibody (that is bound to the protein of interest) was then added. This fluorescence can be visualized and quantified using appropriate equipment and software.

Protocol

Two-layer polyacrylamide gels were cast for protein separation. The top layer [used for initial separation of proteins under lower voltage (80 Volts)] contained 12% acrylamide, 3% bisacrylamide, 1.0% ammonium persulfate and 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED) in deionized

distilled water (ddH_2O). The bottom layer [used for final separation of proteins under higher voltage (120 Volts)] contained 3.6% acrylamide, 1.0% bisacrylamide, 0.4% ammonium persulfate, and 0.03% TEMED in ddH_2O. Mesenteric artery samples were diluted as necessary to generate 1 μg/μL solutions containing 10% β-mercaptoethanol (Sigma). The samples were subsequently placed in boiling water for 5 minutes for further protein denaturation. 15 μL of each sample (15 μg of protein) were loaded into the polyacrylamide gel. Fluorescent and visible protein ladders (Fermentas, Burlington, ON) were loaded to allow for quantification of protein size.

Following separation, the proteins were transferred from the gel on to a nitrocellulose membrane (Bio-Rad, Mississauga, ON) by electrical current (20 Volts) overnight at 4 °C. The membrane was then exposed to 5% skimmed milk in 0.005% Tween™ (polyoxyethylene 20-soribitan monolaurate, Fisher Scientific, Ottawa, ON) at room temperature for 3 hours to reduce non-specific binding of the primary antibody.

For protein detection, membranes were incubated with 1:4000 anti-CYP4A antibody (Affinity BioReagents, Golden, CO) or anti-ET_A receptor antibody (Calbiochem, San Diego, CA) for 3 days at 4 °C. Subsequently, membranes were incubated with peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibody (1:4000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and precision streptactin-horseradish peroxidase conjugate (1:5000, Bio-Rad) for 40 minutes at room temperature. Fluorescence was visualized using ECL™ wstern blotting detector reagents (Amersham, Little Chalfont, UK) and the Fluor-S Max multi-imager (Bio-Rad). This protocol was repeated using an anti-actin antibody (1:4000, Santa Cruz Biotechnologies Inc., Santa Cruz, CA) on the same membranes (following an overnight washout using 0.005% Tween™) to ensure that the same amount of protein was added to each well.

2.2. Messenger RNA Quantification

Isolation of Mesenteric Arteries

Following euthanasia, mesenteric arcades were excised from young (3-4 months) and aged (17-18 months) male, as well as aged (15-16 months) intact, ovariectomized (OVX) and ovariectomized (OVX-E) rats. All the mesenteric arteries were rapidly (< 30 minutes) dissected free of fat and connective tissue, frozen in liquid nitrogen and stored at -80 °C. Kidneys were also collected and frozen in liquid nitrogen to be used as a positive control for CYP4A expression.

RNA isolation

Mesenteric Arteries

The collected arteries were removed from the freezer and immediately immersed in 350 µL of TRIzol™ (Invitrogen, Burlington, ON) to lyse cells and preserve RNA integrity. The samples were homogenized on ice. 70 µL chloroform was added and the samples centrifuged at 11,180 *g* at 4 °C for 15 minutes to separate the samples into organic and aqueous phases. The aqueous (RNA-containing) phase was removed and an equal volume of isopropanol was added. 1 µL glycogen (20 mg/mL; Roche Scientific) and 30 µL sodium acetate (3 µmol/L; Sigma) were added to facilitate precipitation of the RNA. The RNA was kept at -80 °C for 3 days to allow the small amounts of RNA present to precipitate. Subsequently, the samples were thawed and centrifuged at 11,180 *g* for 30 minutes at 4 °C. The resulting pellet was washed with 70% ethanol and air-dried.

The pellet was re-suspended in 10 µL RNAse-free water (Invitrogen). The concentration of total RNA present was determined by measuring the sample absorbance with a spectrophotometer (Hewlett Packard 8452A). Nucleic acids absorb ultraviolet radiation at 260 nm while contaminants (e.g. protein) absorb ultraviolet radiation at 280 nm. Thus, the ratio of the optical density of a sample at 260 nm to that at 280 nm serves as a measure of the purity of the

RNA sample relative to protein contamination. A ratio of 1.7 is indicative of appropriate RNA isolation. All of the samples were subsequently diluted to make 1, 0.5 or 0.25 μg/μL solutions of RNA in RNAse-free water depending on the amount of RNA present in the sample.

Kidneys

As a positive control, frozen kidneys were crushed using a ceramic mortar and pestle on dry ice. Some of the kidney tissue was collected and suspended in 350 μL TRIzol™. The remaining protocol is as above.

Polymerase Chain Reaction

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to quantify the abundance of mRNA sequences for CYP4A genes. The polymerase chain reaction causes exponential amplification of DNA allowing for relative quantification of genetic sequences.[8] Therefore, the RNA samples must be converted to DNA using a reverse transcriptase enzyme. DNA is then heated to generate single-stranded sequences that, upon cooling, anneal to gene-specific primers and are amplified by heat-stable DNA polymerases.[8] Repeated heating and cooling cycles generate double-stranded DNA amplicons that can be detected with SYBR Green (Applied Biosystems, Foster City, CA).[8] SYBR Green fluoresces at 521 nm when bound to double-stranded DNA. Using a Real-Time RT-PCR machine, the fluorescence after each PCR cycle can be measured and a threshold cycle can be determined. That is, the cycle at which the number of amplicons begins to increase exponentially.

Protocol

The appropriate amount of the RNA sample to have one μg for reverse transcription was annealed to random DNA primers (Invitrogen). The cDNA was elongated by incubation with the reverse transcriptase Superscript II (Invitrogen) at 42 °C for one hour in the presence of the RNase inhibitor RNaseOUT (Invitrogen). RNA was also incubated in the absence of

Superscript II to ensure that the DNA detected during the PCR cycles was newly generated and not genomic.

Following reverse transcription (RT), 25 μL PCR reactions were prepared in triplicate for each sample (each containing 3.5 μL RT product, 12.5 μL SYBR Green, 0.5 μL Forward Primer, 0.5 μL Reverse Primer and 10.5 μL ddH_2O). Primer sequences and annealing temperatures are listed in Table 4.1. All primer sequences have previously been used with Real-Time RT-PCR[9-11] Additional samples were subjected to PCR in the absence of RT product to rule out contamination with ambient DNA. PCR plates were placed in an iCycler (Bio-Rad) and the following PCR protocol was used: 1. denaturation of cDNA (95 °C, 10 minutes) 2. 40 cycles of denaturation (95 °C, 20 seconds) followed by annealing (appropriate annealing temperature, 1 minute) 3. The temperature was increased by 0.5 °C every 12 seconds from 55 to 95 °C to determine the melting point of the PCR product and, thus, to confirm that only one product had been generated.

In a separate set of reactions, primer efficiency (the actual amount of amplicon produced compared to the theoretical possible maximum under optimal conditions) was determined by diluting the RT product from a kidney sample and generating a standard curve for each set of primers. The correlation coefficient is a measure of how well the PCR reaction adheres to the expected linear relationship between amount of RT product and double-stranded DNA produced. Correlation coefficients and PCR efficiency for each set of primers are listed in Table 4.2. Since mRNA quantification was sporadic in the test samples (see below), the data were not reported as a ratio of 18S expression according to the Pfaffl method.[12] Rather, they are reported as threshold cycle (not corrected for primer efficiency) to allow for qualitative visualization of the results.

3. Results

3.1. Protein Quantification

Protein was successfully extracted from mesenteric arteries of young and aged rats. Sample protein concentrations are listed in Table 4.3. In mesenteric arterial samples from young male rats, the primary antibody for CYP4A resulted in the detection of an uneven band at around 50 kDa. Representative western blots are depicted in Figure 4.1, Panels A and C. In contrast, on the same membranes, the primary antibody for the loading control protein (smooth muscle actin) resulted in the detection of a smooth and consistent band at 40 kDa (representative blots; Figure 4.1, Panels B and D). Although the bands for CYP4A and actin were at the appropriate molecular weights, the irregular CYP4A band prevented quantification of CYP4A expression in mesenteric arteries. Despite abundant expression of smooth muscle actin, expression of the ET_A receptor was not detectable on the same membranes (Figure 4.1, Panels E and F).

3.2. Messenger RNA Quantification

RNA was successfully isolated from mesenteric arteries and aortae from young and aged male rats as well as aged intact ovariectomized (OVX) and ovariectomized/estrogen-replaced (OVX-E) rats (Table 4.4). Expression of 18-S, CYP4A1, CYP4A2, CYP4A3, CYP4A8, ET_A and actin were detected in the positive control tissue from kidney (data not shown). Furthermore, 18S expression was detected in all of the mesenteric artery and aortic samples from each group (Figure 4.2, Panel A and Figure 4.3, Panel A). However, in mesenteric arteries only CYP4A8 expression was detected and only in samples from young males and OVX females (Figure 4.2, Panel B). Similarly, only CYP4A1 and CYP4A8 were detected in aortic samples, but not from all groups (Figure 4.3, Panels B and C). mRNA for ET_A was only detected in young males (Figure 4.2, Panel C). Since ET_A has been reported to be highly expressed in mesenteric arteries,[13,14] and ET-1 elicited vasoconstriction in our study, we tested the expression of smooth muscle actin as a secondary control. In agreement with our other findings, mRNA for actin was only expressed in only 6 of 21 samples from mesenteric arteries and 5

of 15 aortic samples (Figure 4.2, Panel D and Figure 4.3, Panel D). Accordingly, we were not confident in our ability to reliably quantify CYP4A or ET_A expression using Real-Time RT-PCR.

4. Discussion

Our objectives were to determine: (1) the effect of aging on CYP4A and ET_A expression in mesenteric arteries from young and aged male rats and (2) the effect of ovariectomy ± estrogen replacement on CYP4A expression in mesenteric arteries from aged female rats. However, significant technical limitations prevented us from interpreting our data.

Western blotting with the anti-CYP4A antibody resulted in an uneven, irregularly-shaped band at approximately 50 kDa. Although this molecular weight is consistent with previous reports,[15,16] the patchy nature of the band rendered quantification of expression impossible. Notably, this staining pattern was not observed with the anti-actin antibody. Furthermore, the anti-ET_A antibody did not detect any immunoreactive protein although ET_A expression has been reported in vascular tissue from both young and aged rats.[17] In addition, our functional data illustrated that ET-1 induced potent vasoconstriction while treatment of vessels with the ET_A inhibitor BQ123 reduced their sensitivity to phenylephrine. Combined, these findings suggest that ET_A was present in our vessels but our protocol for western blotting was not effective in quantifying this protein. Further optimization of this protocol is essential for future experiments regarding protein expression in small mesenteric arteries. Particularly, every effort must be made to load the most protein possible into each well. This could be accomplished by reducing the dilution of the samples, or possibly, by pooling the mesenteric arteries from several animals to generate sufficient protein.

Due to these difficulties, our efforts were focused on the quantification of CYP4A and ET_A mRNA expression using Real-Time RT-PCR. We were successful in isolating RNA from mesenteric arteries; however, there was very little expression of CYP4A and ET_A in these samples. In contrast, cDNA for all four isoforms of CYP4A and ET_A were

detected in kidney tissue samples. The primary difference between the protocols for these two types of tissue is the time required to collect them. Kidneys are removed from the animal, washed in HEPES-PSS, and then frozen in liquid nitrogen. Mesenteric arteries, however, need to be dissected free of the surrounding adipose and connective tissue as well as the mesenteric veins before individual arterial segments are frozen in liquid nitrogen. In our laboratory, it takes approximately 25 minutes between euthanasia of the animal and collection of all of the mesenteric arteries. Although precautions were taken (e.g. samples were kept on ice and dissection tools were treated with RNase*Zap*™ [Ambion, Foster City, CA]), RNase-dependent degradation of the sample likely occurred during this time.

Therefore, we tested the expression of CYP4A enzymes in the aortae from the same animals. Aortae are large enough to be dissected macroscopically and immediately frozen. Indeed, some of the aortic samples resulted in the amplification of cDNA for CYP4A1 and CYP4A8. Since the expression of ribosomal 18S was detected in all of the samples, this data suggested that only these two isoforms were present in the aortae of rats. However, since only some of the aortic samples had detectable levels of CYP4A enzymes, we tested the expression of smooth muscle actin as a secondary lower-abundance and smooth muscle-specific control. Actin was detected in approximately 30% of the samples from aortae and from mesenteric arteries. These findings indicated that our protocol, while acceptable for some samples, was not capable of generating data regarding mRNA levels in arterial samples from rats. Accordingly, ET_A expression was not tested in aortae. Further prevention of sample degradation during processing and optimization of the cycling protocol for Real-Time RT-PCR may allow for quantitative detection of CYP4A isoforms and ET_A in the future. Indeed, the length of the amplicons (89-295 base pairs; Table 4.1) generated by the primers used in this study may have reduced amplification during the PCR cycles and thus diminished our ability to determine accurate mRNA expression levels. Thus, primer sequences should be re-visited prior to further investigations.

Importantly, combined with our functional data (see Chapters 2 and 3), our findings

do not indicate that CYP4A and ET_A are not expressed in the vasculature of rats, only that we were unable to detect them.

5. Tables

Table 4.1 Primer information for Real-time RT-PCR.

Gene	Primer Sequences	Amplicon Length (base pairs)	Annealing Temperature (^{O}C)
18S	FW: CGGCTACCACATCCAAGGAA RV: GCTGGAATTACCGCGGCT	186	60.0
CYP4A1	FW: TCCAGGCATTGTCAGAGAACT RV: TAAATGGAGAGTGTGACTTGGATA	89	62.0
CYP4A2	FW: AGATCCAAAGCCTTATCAATC RV: TGATCCTGGTCATCAAGCTTC	210	58.5
CYP4A3	FW: CAAAGGCTTCTGGAATTTATC RV: TGATCCTGGTCATCAAGCTTC	201	58.5
CYP4A8	FW: GGGCATGAGTGGCTCGG RV: GCTGGGTAGCTCTGAGTAACCAT	148	64.5
ET_A	FW: AACCTGGCAACCATGAACTC RV: GCAACAGAGGCATGACTGAA	92	62.0
α-Actin	FW: AGTCGCCATACGGAACCTCGAG RV: ATCTTTTCCATGTCGTCCCAGTTG	295	64.6

Table 4.2 Primer efficiency for Real-Time RT-PCR

Gene	Correlation Coefficient	PCR Efficiency
18S	0.999	83.3%
CYP4A1	0.997	91.1%
CYP4A2	0.998	84.1%
CYP4A3	0.998	85.5%
CYP4A8	0.998	109.9%
ET_A	0.981	97.6%

Table 4.3 **Protein concentrations in samples from mesenteric arteries from young and aged male rats**

Group	[Protein] (μg/μL)
Young Male	2.20
	2.79
	3.56
	4.87
Aged Male	4.68
	6.71
	3.42
	6.48

Table 4.4 **RNA purity[6] and concentrations in samples from mesenteric arteries and aortae from young and aged male rats as well as aged intact, ovariectomized (OVX) and ovariectomized/estrogen-replaced (OVX-E) female rats.**

MESENTERIC ARTERIES				AORTAE			
Group	**Sample Number**	**[RNA] (µg/µL)**	**OD_{260} /OD_{280}**	**Group**	**Sample Number**	**[RNA] (µg/µL)**	**OD_{260} /OD_{280}**
Young Male	A1	1.38	1.84	**Young Male**	A5	0.68	2.07
	A2	0.71	2.00		A6	2.24	2.03
	A3	1.70	1.85		A7	0.61	1.94
	A4	1.11	1.77				
Aged Male	B1	1.09	1.98	**Aged Male**	B6	3.34	1.88
	B2	1.26	1.88		B7	1.80	2.12
	B3	1.81	2.07		B8	4.10	1.81
	B4	2.38	1.86				
	B5	1.52	1.69				
Intact Female	I1	0.86	1.72	**Intact Female**	I5	3.84	1.58
	I2	1.00	1.73		I6	1.475	2.07
	I3	1.12	1.68		I7	2.44	2.09
	I4	2.60	2.28				
OVX Female	O1	0.62	1.84	**OVX Female**	O5	0.97	2.08
	O2	1.20	1.59		O6	3.15	2
	O3	0.65	2.05		O7	2.15	2.08
	O4	0.71	1.70				
OVX-E Female	E1	0.49	1.71	**OVX-E Female**	E5	2.56	2.07
	E2	1.12	n/a		E6	1.33	2.09
	E3	0.54	1.73		E7	1.01	2.06
	E4	0.91	1.62				

[6] RNA purity is determined by exposing the sample to ultraviolet radiation. Nucleic acids have a peak absorption at 260 nm while contaminants (such as proteins) absorb primarily at 280 nm. Thus the ratio of the absorbance at 260 nm to that at 280 nm is considered to be a measure of RNA purity.

6. Figures

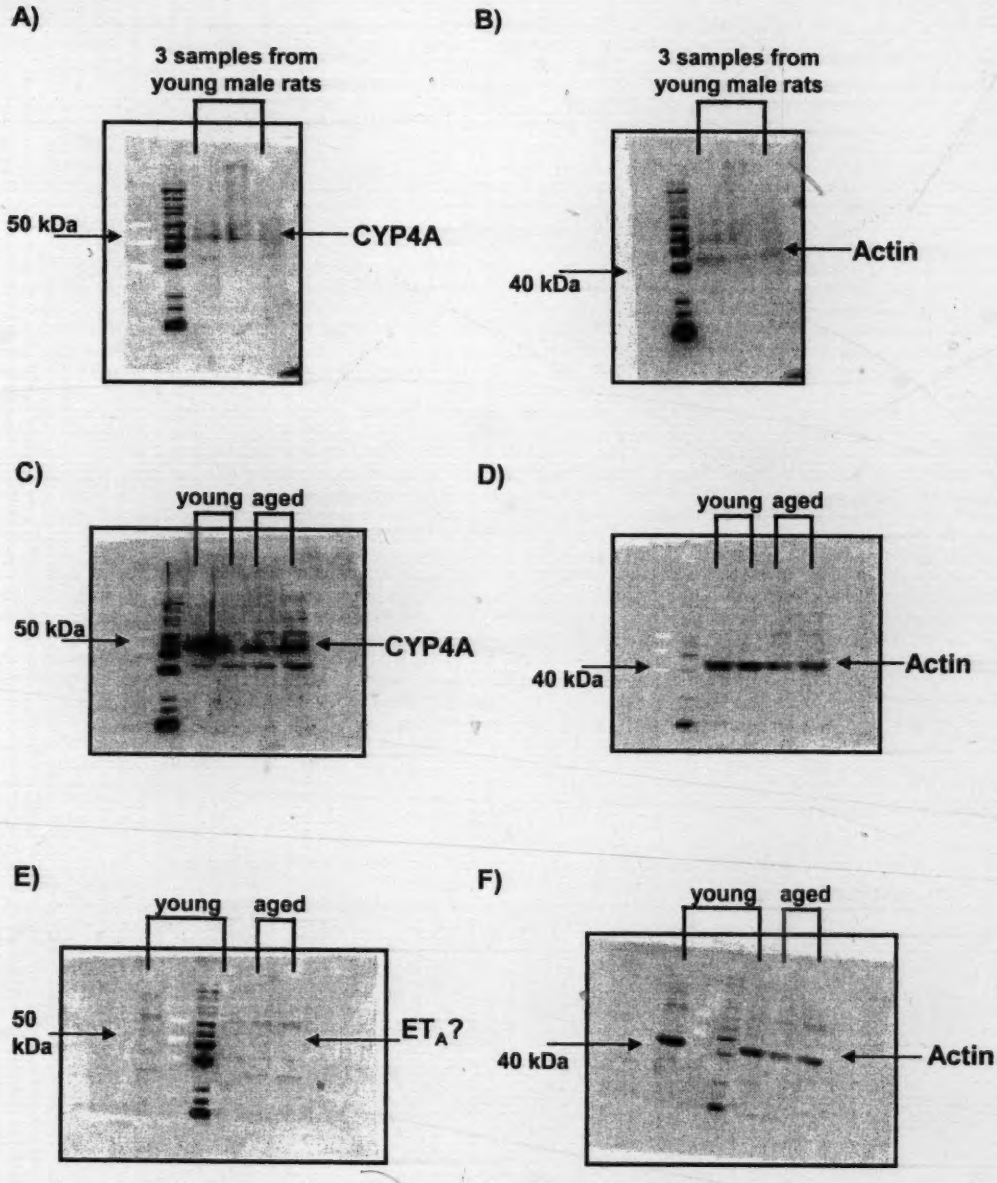

Figure 4.1 Immunostaining for CYP4A and ET_A in mesenteric arteries.

Representative immunoblots depicting expression of CYP4A (Panels **A** and **C**), ET_A (Panel **E**) and their respective loading controls (smooth muscle actin) (Panels **B**, **D** and **F**) in mesenteric artery samples from young and aged male rats.

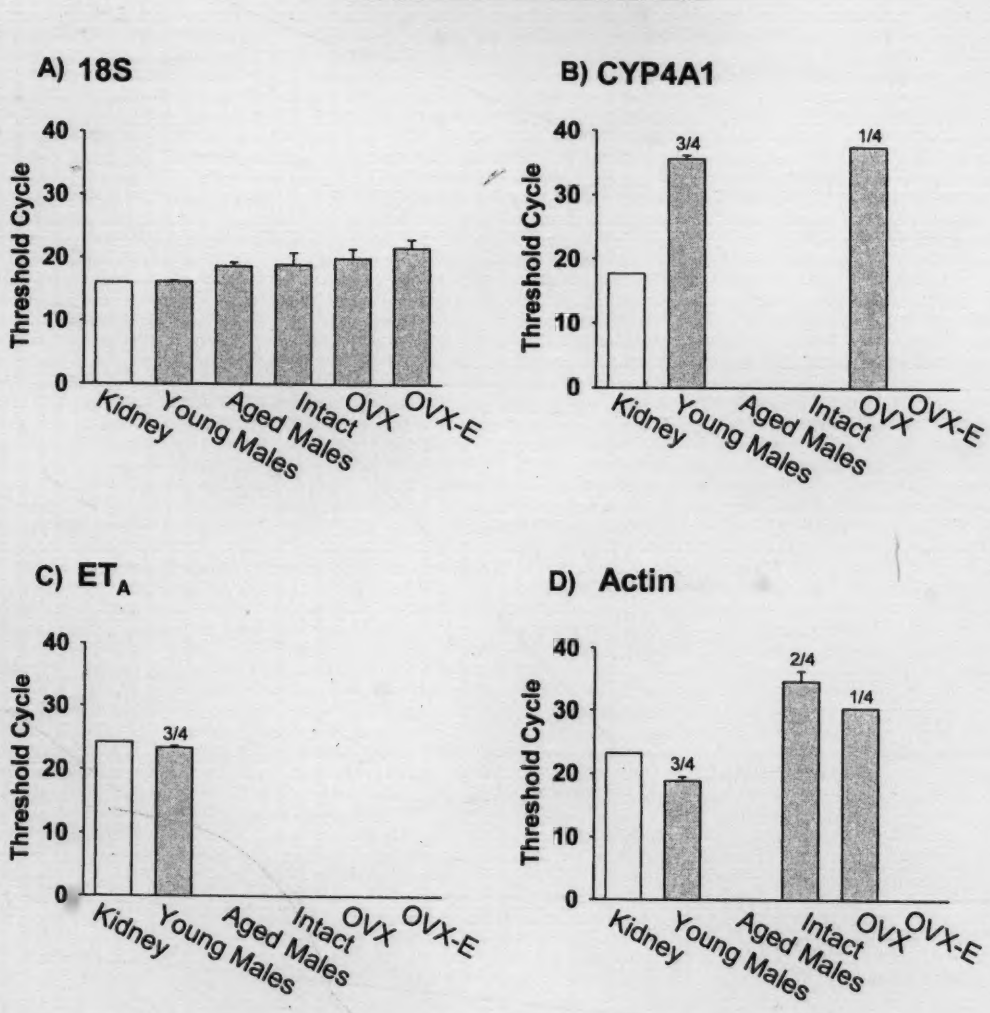

Figure 4.2 Threshold cycles for genes of interest in mesenteric arteries from rats.

Bars represent mean threshold cycles for amplification of cDNA for **A.** ribosomal 18S **B.** CYP4A8 **C.** ET_A receptor and **D.** smooth muscle α-actin in samples derived from kidney (positive control) and mesenteric arteries from young and aged male rats as well as aged intact, ovariectomized (OVX) and ovariectomized/estrogen-replaced (OVX-E) rats ± standard error of the mean (where possible). Where expression was not present in all samples, the number of samples that contained cDNA for the gene of interest out of the total number of samples for that group is depicted above the bars.

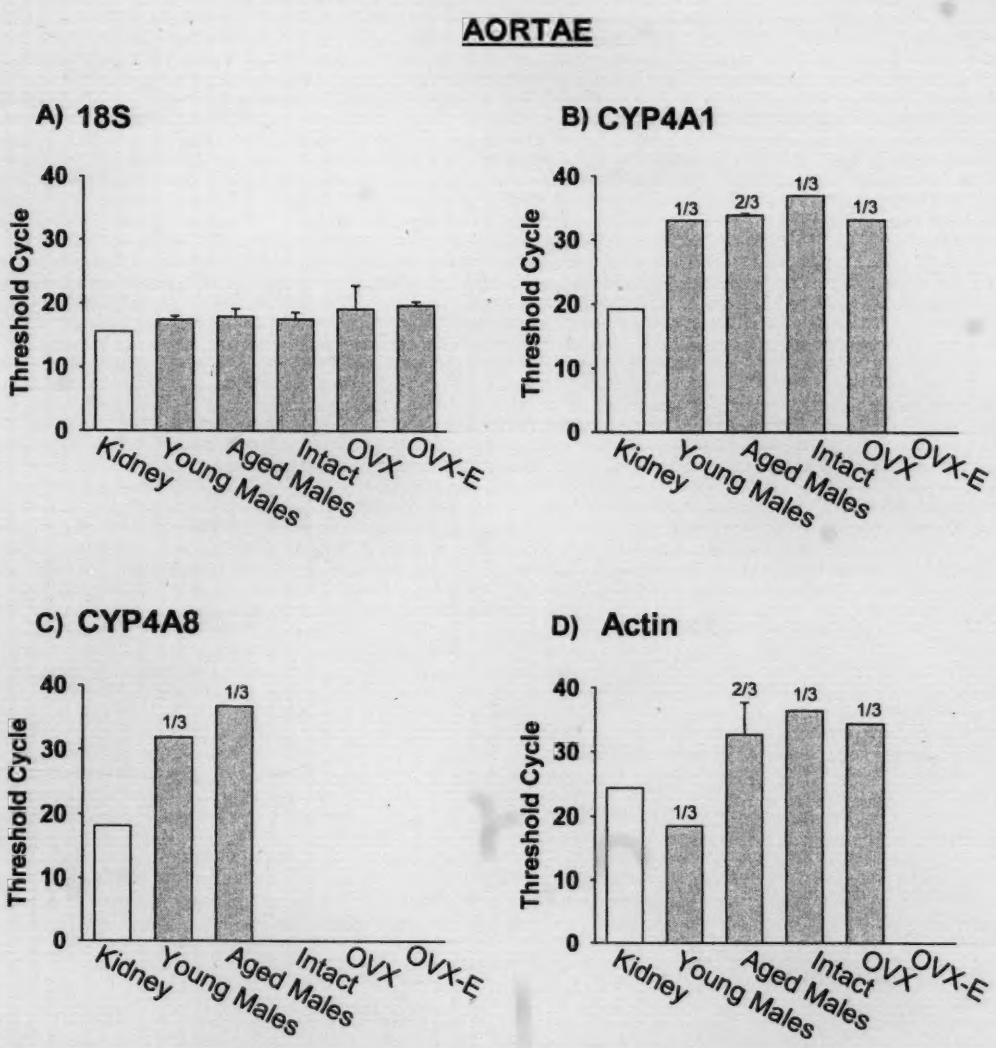

Figure 4.3 Threshold cycles for genes of interest in aortae from rats.

Bars represent mean threshold cycles for amplification of cDNA for **A.** ribosomal 18S **B.** CYP4A1 **C.** CYP4A8 and **D.** smooth muscle α-actin in samples derived from kidney (positive control) and aortae from young and aged male rats as well as aged intact, ovariectomized (OVX) and ovariectomized/estrogen-replaced (OVX-E) rats ± standard error of the mean (where possible). Where expression was not present in all samples, the number of samples that contained cDNA for the gene of interest out of the total number of samples for that group is depicted above the bars.

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CHAPTER V - DISCUSSION

1. The Effects of Aging on CYP4A Modulation of Vasoconstriction

The studies presented in this thesis have provided novel data regarding the role of CYP4A in the peripheral vasculature of aging males and females. Indeed, CYP4A-dependent modulation of α_1-adrenergic vasoconstriction in mesenteric arteries was increased in aged male rats relative to young and in aged, ovarian hormone-depleted female rats (model of postmenopausal women) relative to their young counterparts. We have further demonstrated that estrogen replacement does not normalize the role of CYP4A in ovariectomized females suggesting that the effects of ovariectomy on CYP4A modulation of vasoconstriction are estrogen-independent. Interestingly, CYP4A did not modulate ET-1-dependent constriction in arteries from male rats or pressure-dependent constriction in arteries from female rats. These data may suggest that CYP4A is specifically activated by α_1-adrenergic stimulation. Overall, despite our inability to quantify CYP4A protein or mRNA expression (see Chapter 4), our data indicate that the contribution of CYP4A to α_1-adrenergic constriction is increased in aging males and females.

1.1. Possible Mechanisms of Aging-Related Changes in CYP4A Modulation of Vasoconstriction

The mechanisms through which aging alters the role of vascular CYP4A still need to be explained; however, aging is known to be associated with increased oxidative stress in the peripheral vasculature.[1] Due to the rapid reaction of NO with superoxide,[2] increased oxidative stress can reduce vascular NO availability. NO has been shown to irreversibly bind to the iron moieties of hemoproteins including CYP enzymes.[3] Moreover, NO binding inhibits hydroxylation by CYP enzymes,[3] and CYP4A activity in the renal vasculature.[4] Therefore, reduced bioavailability of NO in aging may increase CYP4A activity due to reduced NO-dependent inhibition of these enzymes. Oxidative stress is increased in postmenopausal women[5] and ovariectomized rats[6]. Therefore, ovarian hormone deficiency is also likely to result in reduced NO availability. Accordingly, reduced NO-dependent inhibition of CYP4A

may account for the ovariectomy-induced increase in CYP4A modulation α_1-adrenergic vasoconstriction observed in our study.

Aging and hormonal status are also known to affect several of the reported downstream targets of the CYP4A product, 20-HETE. As discussed in chapter 2, BK_{Ca} channel expression is reduced in aging.[7] Furthermore, a recent study has indicated that the contribution of Rho-kinase to cutaneous vasoconstriction is increased in aged, relative to young, humans.[8] In contrast, functional L-type voltage-gated calcium channel activity in mesenteric arteries is not affected by aging in mice.[9] Therefore, CYP4A-generated 20-HETE may have a larger effect in vessels from aged animals due to the reduced number of sites at which it can act to prevent cellular hyperpolarization (BK_{Ca} channels) and increased Rho-kinase activity. In young animals, ovariectomy increases vascular expression of L-type voltage-gated calcium channel subunits and replacement with 17β-estradiol normalizes this expression.[10] Neither ovariectomy nor estrogen replacement affected BK_{Ca} channel expression.[10] In addition, Rho-kinase inhibition resulted in greater vasodilation in ovariectomized rats relative to intact or estrogen-replaced rats.[11] Therefore, Rho-kinase activity may be tonically inhibited by estrogen in young female rats. The effects of menopause or ovariectomy in aged rats have not been determined; however, alteration of vascular ion channels may indeed account for the increase in CYP4A modulation of vasoconstriction in aging female rats.

In the SHR, reduced generation of vasodilatory eicosanoids by CYP2E1 results in increased 20-HETE-dependent sensitization of renal arterioles to phenylephrine-induced vasoconstriction.[12] Therefore, an aging- or ovarian hormone deficiency-related decrease in the production of vasodilatory compounds by other CYP enzymes may reduce endogenous antagonism of 20-HETE activity and thus increase its contribution to vasoconstriction. In addition, the 20-HETE metabolism may result in the generation of vasoactive

molecules.[13] Although, the endogenous catabolic products of endogenous 20-HETE have not yet been shown to play a major role in the control of vascular function, these pathways have not yet been investigated in aging. Thus, further study of this area may be of significant importance in understanding the role of the CYP4A/20-HETE pathway in aging.

1.2. Hormonal Regulation of CYP4A-Dependent Modulation of Vasoconstriction.

Our data from aged female rats (Chapter 3) illustrated that ovariectomy increased CYP4A modulation of α_1-adrenergic vasoconstriction. Furthermore, estrogen replacement in these animals had no additional effect on CYP4A. However, our study did not rule out the effect of other ovarian hormones including progesterone and androgens on vascular CYP4A. These hormones have previously been implicated in the regulation of CYP enzymes. Progesterone metabolites can activate the pregnane X receptor (PXR) resulting in the activation of CYP3A4.[14] Furthermore, PXR activation during pregnancy may increase vasodilation due to activation of CYP expoxygenase acivity.[15] In contrast, androgen treatment has been associated with increased CYP4A expression, 20-HETE formation, and blood pressure in male and female rodents.[16,17] Interestingly, androgen production is regulated by aging in men[18] and women.[19] In aging men[20] and rats,[21] androgen secretion is reduced over time. Similarly, androgen production is reduced in postmenopausal and ovariectomized women.[19] Thus, the increase in CYP4A modulation of α_1-adrenergic vasoconstriction in aged male rats (relative to young rats) and aged OVX and OVX-E rats (relative to aged intact rats) observed in our studies is not likely to be mediated by stimulation of CYP4A by androgens. Overall, determining whether or not vascular CYP4A is hormonally regulated in aging males and females will require future investigation.

1.3. CYP4A and α_1- Adrenergic Receptors

In our studies, phenylephrine-dependent vasoconstriction was mediated by CYP4A in aged male rats and ovarian hormone-deficient aged female rats. By binding to α_1 adrenergic receptors, phenylephrine can activate PLD.[22] PLD can then act to liberate AA from the plasma membrane to act as substrate for CYP4A-dependent formation of 20-HETE. A similar pathway has been observed in renal arteries whereby ET-1 activates both PLA_2[23] and PLC.[24] These enzymes liberate AA from the plasma membrane and have been demonstrated to result in 20-HETE formation.[25] Therefore, we expected that CYP4A would also modulate ET-1-dependent vasoconstriction in mesenteric arteries. In contrast, ET-1-dependent constriction was not affected by CYP4A inhibition in young or aged rats However, data regarding ET_A inhibition suggested that endogenous CYP4A and ET_A act in concert in aging. Thus, it is possible that exogenous ET-1-dependent constriction does activate CYP4A production of 20-HETE, but because ET-1 is the most potent known vasoconstrictor,[26] this activation is not a critical step in the generation of vasoconstriction. It will be important to determine if constriction induced by other agonists (such as the thromboxane A_2 agonist U46619) is affected by CYP4A inhibition in aged male rats. Further studies will be required to substantiate our finding that CYP4A activation may be α_1-adrenergic receptor-specific in mesenteric arteries from aged rats and, if so, the mechanisms that underlie this specificity.

Numerous studies have reported that myogenic constriction is reduced by CYP4A inhibition in various vascular beds in male animals.[27-30] However, in our study, myogenic constriction was not affected by CYP4A inhibition in arteries from intact, OVX, or OVX-E animals. Profound gender differences in the generation and modulation of myogenic constriction have been reported in mesenteric,[31] cerebral,[32] and skeletal muscle[33] arteries. Aging can also alter the regulation of myogenic constriction in small arteries from rats[34] and humans.[35] Thus, gender- and/or aging-induced changes in the mechanisms

underlying myogenic tone development may explain why myogenic tone was not CYP4A-dependent in our aged female rats. In addition, increased intraluminal pressure results in depolarization of vascular smooth muscle that is believed to result from activation of mechanosensitive ion channels or transporters.[36] Thus, myogenic tone development does not entirely depend on activation of G-protein coupled receptors (such as α_1-adrenergic or ET_A receptors). It will be important to study the role of CYP4A in myogenic tone development in mesenteric arteries from young female rats in order to determine if this role is gender- or gender and age-dependent.

Interestingly, arterial sensitivity to phenylephrine-dependent vasoconstriction has largely been reported to be reduced in aged animals [37,38] and humans.[39,40] Furthermore, release of calcium from the sarcoplasmic reticulum through IP_3 and ryanodine receptors in response to phenylephrine stimulation is reduced in smooth muscle cells from mesenteric arteries of aged male mice.[9] However, arterial sensitivity to α-adrenergic vasoconstriction has also been reported to be unchanged[41] or increased[42] in aging. In our study, phenylephrine-dependent vasoconstriction was not affected by age in male rats. In female rats, simulation of menopause increased phenylephrine-dependent constriction. Since CYP4A inhibition reduced vasoconstriction in arteries from aged rats of both genders, our data suggest that CYP4A-dependent production of 20-HETE supports α_1-adrenergic vasoconstriction in aged males and increases this constriction in aged ovarian hormone-deficient females.

1.4. Limitations

The data presented in this thesis may be of considerable interest in furthering the understanding of the vascular CYP4A pathway in aging male and female rats. There are, however, several limitations of this work.

Firstly, it is important to consider the validity of studying aging male and female rats as a model of aging men and women. Indeed, aging rats continue to grow in length throughout their lifetime and are allowed *ad libitum* access to food (for their entire life in males and until ovariectomy in females). Furthermore, female rats do not undergo menopause thus requiring surgical intervention to model postmenopausal women. Thus this model is limited by both physiological and environmental factors.

In addition, although the CYP4A pathway contributes to vasoconstriction in the peripheral vasculature, CYP4A activity in the kidney has potent anti-hypertensive properties (e.g. inhibition of sodium reabsorption, see Chapter 1, section 5.2).[43,44] Thus, any future therapeutic interventions designed to diminish aging-related increases in CYP4A-mediated vasoconstriction would have to be targeted specifically to the peripheral vasculature.

The data regarding the involvement of CYP4A in vasoconstriction is dependent upon the use of DDMS. DDMS is the only widely-available specific inhibitor of ω-hydroxylase activity;[13] however, the possibility of it having non-specific effects cannot be ignored. Furthermore, our preliminary experiments suggested that exogenous 20-HETE had no effect on vascular tone in arteries from female rats. However, this series of experiments was not completed due to technical difficulties regarding the myograph equipment. Thus, the effect of 20-HETE on mesenteric arteries in this model remains to be determined.

Finally, due to technical difficulties resulting largely from the size of mesenteric arteries, arterial mRNA and protein expression could not be quantified. This may be possible in future investigations pending revision of the real-time RT-PCR protocol and recent improvements in the specificity of CYP4A antibodies (personal communication with Dr. John Seubert).

1.5. Future Directions

There are many avenues of investigation for the vascular CYP4A pathway. Firstly, it will be important to determine if basal production of 20-HETE is affected by aging in mesenteric arteries from male rats. When our study was conducted (see Chapter 2), it was believed that exogenous AA was required to provide sufficient substrate for the production of quantifiable 20-HETE formation. Recently, however, Dr. Roman's laboratory (Medical College of Wisconsin, Milwaukee, WI) has demonstrated that arterial production of quantifiable amounts of 20-HETE is possible in the absence of any exogenous AA. Thus, it will be possible to determine how aging affects basal and agonist-induced production of 20-HETE. This will facilitate characterization of the rate-limiting steps in CYP4A production 20-HETE (e.g. availability of AA) and further our understanding of this pathway in aging.

In addition, optimization of the protocols for CYP4A protein and mRNA quantification will provide valuable insight regarding the expression CYP isoforms in aging. Accordingly, it will be possible to determine if the increased CYP4A modulation of vasoconstriction is due to a difference in CYP4A expression and/or activity. Further functional studies regarding the downstream effectors of CYP4A-dependent 20-HETE activity will also be of substantial interest in further elucidating the effects of aging on CYP4A modulation of vascular function.

1.6. Conclusion

In summary, the data presented in this thesis have suggested an important role for CYP4A in the modulation of α_1-adrenergic vasoconstriction in aged male rats and aged ovariectomized female rats. Aging-related induction of the CYP4A vasoconstrictor pathway may thus contribute to altered peripheral vascular function in aging men and women. Therefore, future study of this pathway may provide novel, gender-independent, targets for the prevention and management of cardiovascular disease in our aging population.

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